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(54) Title: SUGAR BEET GENES INVOLVED IN STRESS TOLERANCE

(57) Abstract: The present invention relates to isolated genes originating from *Beta vulgaris*, sugar beet, that are involved in responses to stress situations. The genes were isolated from a sugar beet cDNA library screened in a functional selection procedure with transformed yeast cells that were able to grow in selection medium with high salt concentrations. Subsequently these genes were sequenced and further characterized. One of the genes is a sugar beet casein kinase a \$(g)a\$-subunit, one is a sugar beet dihydroorotase, one is a sugar beet translation initiation factor 1A and two others are of a unknown protein type. All of these isolated plant genes were functional as stress tolerance enhancers in yeast cells and are therefore useful to confer stress tolerance to an organism when transfected herein. More particularly, these genes can be used to render crops resistant to stress situations like osmotic stress caused by salt, drought, cold or frost.

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## SUGAR BEET GENES INVOLVED IN STRESS TOLERANCE

### FIELD OF INVENTION

- 5 The present invention relates to the field of plant molecular biology, more particularly to the use of sugar beet genes and proteins, able to confer a phenotype in eukaryotic cells or organisms of tolerance to stress situations for example mineral salt toxicity caused by ions such as Na<sup>+</sup> or Li<sup>+</sup>.

### BACKGROUND

- 10 Soil salinity is one of the most significant abiotic stresses for plant agriculture and therefore it would be useful to identify and isolate stress tolerance genes for the practical goal of genetically improving the salt tolerance of crop plants.

Two other major abiotic stresses, drought and cold, are intimately linked with salt stress. Many genes that are regulated by salt stress are also responsive to drought or cold stress  
15 (Zhu, 1997), therefore these genes are particularly interesting for genetically improving of stress tolerance.

- The molecular mechanisms by which plants respond to salt stress are starting to be elucidated (Hasegawa *et al.* 2000a). The sodium transporters at the vacuole and plasma membrane, identified as the products of the Arabidopsis *NHX1* (Gaxiola *et al.* 1992, Apse  
20 *et al.* 1999) and *SOS1* (Shi *et al.* 2000) genes respectively, have been described as important determinants of salt tolerance.

- For the goal of genetically improving stress tolerance in plants it is important to use stress tolerance genes that when introduced can immediately confer stress tolerance. The action of these genes cannot be dependent on other pathway-related events or other  
25 components that are necessary for the molecular mechanism of stress tolerance. One can identify important stress factors in the stressed organism, but the question remains whether these genes will also contribute in enhancing stress tolerance in a heterologous host when isolated and transfected herein.

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### Sugar beet (genes) and stress

- It is known that *Beta vulgaris* L. (*Chenopodaceae*, sugar beet), is rather a stress resistant plant when compared to other plants e. g. *Arabidopsis thaliana*. Sugar beets are relatively spoken rather stress resistant to salt- and drought. The genes that are responsible for the  
35 ability of sugar beet to grow in more difficult conditions are started to be elucidated. A first indication that a gene under investigation might be involved in the induction of resistance, is the increase of its expression under stress conditions. As an example, Matthias *et al.*

(1996) showed that salt stress induces the increased expression of V-type H<sup>+</sup>-ATPase in mature sugar beet leaves. Also betaine, an osmoprotectant, is accumulated by many beet plants in response to salinity and drought. Furthermore in sugar beet, the expression of Choline monooxygenase, which catalyzes the committing step in the synthesis of glycine betaine of sugar beet, is also induced by osmotic stress in *Chenopodaceae*. The mRNA levels in leaves increased 3- to 7- fold at 400mM salt and returned to uninduced levels (Russel, 1998). As mentioned above there are several alternative pathways to respond to stress situations and therefore many different genes are probably involved in stress responses.

## 10 SUMMARY OF THE INVENTION

In the present invention five novel genes were isolated from *Beta vulgaris* and were transformed to yeast cells. These genes all induced stress tolerance in the yeast cells, and encode for a casein kinase  $\alpha$  subunit, referred to as BvCK2A, a dihydroorotase, referred to as BvDHO, a translation initiation factor 1A, referred to as Bvelf-1A, and for two other unknown proteins, further referred to as Bv120 and Bv20Li.

Unexpectedly, the inventors demonstrated that these sugar beet genes when transformed in yeast could render this heterologous organism tolerant to stress, Na<sup>+</sup> stress in particular. The surprisingly strong phenotype of some of these yeast clones and the fact that these genes in an isolated position and in a heterologous background acted as stress tolerance enhancers, makes these genes very attractive tools to induce stress tolerance in any organism of interest, without the need for accessory compounds. The ability of these genes to enhance osmotic stress tolerance in yeast cells when isolated and transfected herein, clearly distinct them from other sugar beet genes that are known to play a role in (other) stress responses, but that were not used in an isolated form. One of these novel sugar beet genes of the present invention encodes a subunit of casein kinase. It was surprising to disclose for the first time a plant casein kinase subunit and more surprisingly an  $\alpha$  catalytic subdomain of casein kinase exerting a function in salt stress response. Herein is described a cDNA clone (*BvCKA2*) encoding one of the catalytic  $\alpha$  subunits of sugar beet protein kinase CK2 (formerly casein kinase II). *BvCKA2* increases the tolerance to NaCl of yeast. In addition, it is herein shown that expression of *BvCKA2* in sugar beet is induced by salt stress.

Also the identification of two other genes as a sugar beet dihydroorotase and a translation initiation factor 1A, respectively, was very surprising since, in the present state of the art of the plant-stress field, there is no evidence for the function of such proteins as stress tolerance inducers.

Furthermore it was surprising that two of the isolated genes encoded polypeptides for which no homologues could be found. Therefore these genes can be considered as a novel type of genes encoding a novel type of protein that confer to a heterologous organism tolerance to stress.

- 5 All these genes of the present invention are cloned in an expressible vector format and are able to contribute agronomically interesting features to a transgenic plant when transfected herein.

To identify novel plant genes involved in NaCl stress, the inventors adopted a strategy previously utilized with the yeast *Saccharomyces cerevisiae* (Serrano, 1996). The  
10 inventors constructed a cDNA library from salt-stressed sugar beet and screened it in a salt-sensitive yeast strain. The rational for this screening of plant genes expressed in yeast cells is that some of the molecular mechanisms of yeast salt tolerance are thought to be similar to those of plant cells (Serrano, 1996, Hasegawa *et al.* 2000b). Sugar beet is a relatively halophytic crop plant (Marschner, 1995) which could be a better source of  
15 halotolerance genes than the model plant *Arabidopsis*.

Accordingly, the invention embodies five novel *Beta vulgaris* genes with nucleotide sequences as given in SEQ ID NOs 1 to 5, encoding five different polypeptides with amino acid sequences as given in SEQ ID NOs 6 to 10.

- The present invention further relates to vectors, or host cells or organisms comprising at  
20 least part of the sequences as set forth in SEQ ID NOs 1 to 5.

Furthermore in another preferred embodiment of the invention, a method is provided for conferring stress tolerance to an organism of interest, preferably plants, yeast or bacteria, comprising the introduction of at least one of the five sugar beet genes in that organism.

#### DETAILED DESCRIPTION OF THE INVENTION

- 25 One of the problems underlying the present invention is to provide genes that can be used to enhance stress tolerance of organisms that suffer from stress conditions like osmotic stress, caused by salt or drought and/or stress conditions like cold, chilling and freezing stress or oxidative stress.

- 30 This invention offers solutions to the above-described problem and is disclosed in the following embodiments characterized in the claims.

A solution is achieved by providing a set of genes that are originating from *Beta vulgaris*, a stress tolerant crop plant, that were isolated and that confer to *Saccharomyces cerevisiae* tolerance to stress conditions, for instance genes that confer stress tolerance to the Na+  
35 sensitive yeast strain JM26. Additional to the fact that these genes are all from the same plant, they all showed a salt resistance phenotype when separately transformed to a salt sensitive yeast mutant. By doing so they all acted as a sole enhancer of stress tolerance.

Unexpectedly these genes encode proteins with very different putative functions like casein kinase  $\alpha$  subunit, dihydroorotase, translation initiation factor 1A and some of them are even of an unknown type. Other features such as the very strong salt resistance phenotype that some of these genes showed in yeast and the fact that some of these genes are frequently isolated in the selective screening procedure in yeast, contribute to the effectiveness and efficiency of these genes as stress tolerance enhancers. This set of genes enables the person skilled in the art to genetically alter the organism of interest in order to make it tolerant to stress situations. For the cultivation of crop plants for example, of which many are sensitive to stress conditions like salt, drought or cold, the disclosed genes offer the possibility to solve the problem of reduced yield and reduced economic profit. Each gene of this set of genes enables the person skilled in the art to modify cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology by introducing at least one of these genes into the cell.

Casein kinase, CK2 (formerly CKII), is a serine-threonine protein kinase, ubiquitous and highly conserved among eukaryotic organisms (Glover, 1998). It is composed of two catalytic subunits ( $\alpha$  or  $\alpha'$ ) and of two regulatory subunits ( $\beta$ ), which tetramerize to adopt an  $\alpha_2\beta_2$  structure. Protein kinase CK2 lokalizes both in the nucleus and in the cytoplasmic compartment where it phosphorylates a variety of substrates involved in different cellular functions. In yeast, CK2 is essential and required for at least four biological processes: flocculation, cell cycle progression, cell polarity and ion homeostasis. In plants, CK2 is proposed to be involved in the regulation of cell cycle (Espunya *et al.* 1999), in the light regulation of plant development (Lee *et al.* 1999) and in the circadian clock function (Sugano *et al.* 1998, Suggano *et al.* 1999). A link between the ectopic expression of plant casein kinase  $\alpha$  subunit and stress in general has not been demonstrated previously.

Accordingly, the invention relates to a novel nucleic acid of sugar beet as set forth in SEQ ID No. 1, further referred to as clone 154, encoding a casein kinase  $\alpha$  catalytic subunit, further referred to as BvCKA2 and capable of enhancing salt tolerance in salt sensitive yeast cells. The open reading frame, starting at nucleotide position 202 and ending at 1203 encodes the BvCKA2 amino acid sequences set forth in SEQ ID NO. 6.

Also the identification of one of the genes as a sugar beet dihydroorotase was very surprising since, in the present state of the art of the plant-stress field, there is no evidence for the function of such an enzyme as a stress tolerance inducer.

Accordingly, the invention relates to a novel nucleic acid of sugar beet as set forth in SEQ ID No. 2, further referred to as clone 35, encoding a dihydroorotase, further referred to as BvDHO and capable of enhancing salt tolerance in salt sensitive yeast cells. The open

reading frame, starting at nucleotide position 199 and ending at 1236 encodes the BvDHO amino acid sequences set forth in SEQ ID NO.7.

Furthermore the isolation of a translation initiation factor 1A of sugar beet, that acts as a sole stress tolerance enhancer, was very surprising. Although it is known that cells  
5 respond to stress by altering the phosphorylation of transcription factors in order to alter the translational capacity, this is the first time that one could demonstrate that a translation factor on its own could contribute in enhancing stress tolerance in an organism when transfected herein.

Accordingly, the invention relates to a novel nucleic acid of sugar beet as set forth in SEQ  
10 ID No. 3, further referred to as clone 76, encoding a translation initiation factor 1A, further referred to as BvelF-1A capable of enhancing salt tolerance in salt sensitive yeast cells. The open reading frame, starting at nucleotide position 88 and ending at 521 encodes the BvelF-1A amino acid sequences set forth in SEQ ID NO. 8.

Two other nucleic acids encoded polypeptides with unknown function were found to confer  
15 stress tolerance in yeast.

Accordingly, the invention relates to a novel nucleic acid of sugar beet as set forth in SEQ  
ID No. 4, further referred to as clone 120, encoding a unknown protein, further referred to  
as Bv120, capable of enhancing salt tolerance in salt sensitive yeast cells. The open  
reading frame, starting at nucleotide position 29 and ending at 499 encodes the Bv120  
20 amino acid sequences set forth in SEQ ID NO. 9.

Furthermore, the invention relates to a novel nucleic acid of sugar beet as set forth in SEQ  
ID No. 5, further referred to as clone 20Li, encoding an unknown protein, further referred  
to as Bv20Li, capable of enhancing salt tolerance in salt sensitive yeast cells. The open  
reading frame, starting at nucleotide position 1 and ending at 879 encodes the Bv20Li  
25 amino acid sequences set forth in SEQ ID NO. 10.

A first aspect of the present invention is the procedure of screening a cDNA library from  
NaCl-induced sugar beet leaves and subsequent isolation of the five sugar beet genes as  
mentioned above. A functional approach to identify sugar beet proteins that are involved in  
the response of plants to salt stress was followed. For this purpose a NaCl-induced cDNA  
expression library was constructed from sugar beet leaves as described in example 1 and  
example 3 and the Na<sup>+</sup>-sensitive yeast mutant strain JM26 (see example 2) was used to  
screen for sugar beet cDNAs that increased the yeast salt tolerance upon overexpression.  
The growth of this yeast mutant is normally inhibited at NaCl concentrations (150 mM)  
similar to those impairing growth of most crop species. This screening procedure is  
35 described in example 4. After transforming 100.000 individual cells with the plasmids  
pYPGE15 containing the cDNA inserts, colonies were pooled and selected for their ability

to grow in the presence of 150 mM NaCl (see example 4). Four of the positive clones, were named clone 154, clone 35, clone 76 and clone 120.

The yeast clones 154, clone 35, clone 76 and 120 had a clear salt tolerance phenotype and the phenotype was very reproducible: under 150 mM NaCl control yeast cells did not grow at all, and yeast cells overexpressing the insert 154, 35, 76 or 120 grew. Clones 35 and 76 were selected twice and three times respectively during the selection of salt tolerant yeast clones, suggesting the abundant presence of those clones in the salt stressed sugar beet leaves.

The same selective screening procedure was also performed to select Li<sup>+</sup> tolerant yeast cells. After transformation of the sugar beet cDNA library, colonies were pooled and selected for their ability to grow in the presence of 20 mM LiCl without methionine as described in example 4. One of the positive clones was named 20Li. Clone 20Li showed a strong Li<sup>+</sup> tolerance phenotype.

The definition of a strong phenotype is based on drop test experiments. Several dilutions of saturated cultures (1:10, 1:100, 1:1000) were made and these were grown on selective media (150 mM NaCl plus methionine or 20 mM LiCl without methionine). Strong phenotypes are those clones that grew well in all the dilutions assayed (e.g. clone 35, 76, 120 for NaCl and clone 20Li for LiCl). Clone 154 had not such a very strong phenotype in yeast because only the first dilution (1:10) was able to grow in selective medium. The control cells expressing the empty plasmid did not grow at all in the selective media.

Accordingly, a first embodiment of the present invention relates to a method for induction of stress tolerance in an organism comprising expression of at least one *Beta vulgaris* gene which confers stress tolerance to yeast cells.

In another embodiment, the invention relates to the use of a *Beta vulgaris* nucleic acid for enhancing stress tolerance in a plant comprising expression of said *Beta vulgaris* nucleic acid characterized in that it confers stress tolerance to yeast cells, for instance yeast cells derived from the Na<sup>+</sup> sensitive yeast strain JM26.

In a further embodiment, the present invention is a method for induction of osmotic stress tolerance in an organism comprising expression of at least one *Beta vulgaris* gene which confers osmotic or oxidative stress tolerance, such as salt stress or drought stress or frost stress tolerance, to yeast cells.

The expression "induction of stress tolerance" as used herein has the same meaning as "enhancing stress tolerance" and therefore can be used interchangeable.

All the selected clones are hereunder described in more detail. To find possible homologues, the amino acid sequence of the (putative) ORFs were subjected to a homology search, performed with the BlastP 2.0.10 program (Altschul *et al.* 1997).

The cDNA insert of the plasmid present in clone 154 contains a 1527 base pair cDNA (SEQ ID No. 1) with an open reading frame of 999 base pairs encoding a polypeptide of 333 amino acids (SEQ ID NO. 6) with a predicted molecular weight of 39.4 kD. This polypeptide, named BvCKA2, has 91,6 % identity with one of the catalytic subunits (alpha chain 2) of the protein kinase CK2 from *Zea mays* (ZMCKA2). BvCKA2 contains the 11 typical subdomains of the eukaryotic protein kinases (Hanks *et al.* 1995) and all the conserved amino acid residues characteristic of CK2 catalytic subunits (Figure 1). The 170-DWG-172 present in the catalytic site is an invariant finger-printing pattern for CK2 alpha subunits (Niefind *et al.* 1998). Also present in BvCKA2 are the essential catalytic lysine 63-K and the highly basic region 69-KKKKIKR-75. The cDNA insert of the plasmid present in clone 35 contains a 1743 base pair cDNA (SEQ ID No. 2) with an open reading frame of 1035 base pairs encoding a polypeptide of 345 amino acids (SEQ ID NO. 7) with a predicted molecular weight of 40.8 kD. This polypeptide, named BvDHO, has 79 % identity with the precursor of the protein dihydroorotase from *Arabidopsis thaliana* and has 81% identity with the Dihydroorotase of potato (WO0118190, WO0114569) The cDNA insert of the plasmid present in clone 76 contains a 643 base pair cDNA (SEQ ID No. 3) with an open reading frame of 432 base pairs encoding a polypeptide of 144 amino acids (SEQ ID NO. 8) with a predicted molecular weight of 17 kD. This polypeptide, named BvelF-1A, has 88% identity with the precursor of the eukaryotic translation initiation factor 1A (eIF-1A or formerly known as eIF-4C) from *Onobrychis viciifolia* (common sainfoin). The cDNA insert of the plasmid present in clone 120 contains a 845 base pair cDNA (SEQ ID No. 4) with an open reading frame of 468 base pairs encoding a polypeptide of 156 amino acids (SEQ ID NO. 9), named Bv120, with a predicted molecular weight of 18.5 kD. The cDNA insert of the plasmid present in clone 20Li contains a 879 base pair cDNA (SEQ ID No. 5) with a putative open reading frame of 876 base pairs encoding a polypeptide of 292 amino acids (SEQ ID NO. 10) with a predicted molecular weight of 34.5 kD. This polypeptide, named Bv20Li, has 59% identity with a predicted protein from a genomic clone of *Arabidopsis thaliana*, for which no function has been described.

In a further embodiment the invention thus relates to the use of a *Beta vulgaris* nucleic acid for enhancing osmotic or oxidative stress tolerance in a plant wherein said *Beta vulgaris* nucleic acid is selected from one of the following:

- (a) a nucleic acid comprising a DNA sequence as given in any of SEQ ID NOs 1 to 5 or the complement thereof,
- (b) a nucleic acid comprising the RNA sequence corresponding to any of SEQ ID NOs 1 to 5 or the complement thereof,
- (c) a nucleic acid specifically hybridizing to the nucleic acid of (a) or (b) under high stringency conditions,



- (d) a nucleic acid encoding a protein with an amino acid sequence which is at least 93%, preferably at least 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 6,
- 5 (e) a nucleic acid encoding a protein with an amino acid sequence which is at least 80%, preferably at least 82%, 85% or 90%, more preferably at least 95%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 7,
- (f) a nucleic acid encoding a protein with an amino acid sequence which is at least 89%, preferably 90%, 92%, 95% or 96%, more preferably 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 8,
- 10 (g) a nucleic acid encoding a protein with an amino acid sequence which is at least 75%, preferably 80%, 85% or 90%, more preferably 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 9,
- (h) a nucleic acid encoding a protein with an amino acid sequence which is at least 65%, preferably 70%, 75%, 80%, 85% or 90%, more preferably 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 10,
- 15 (i) a nucleic acid encoding a protein comprising the amino acid sequence as given in any of SEQ ID NOs 6 to 10,
- (j) a nucleic acid encoding an immunologically active and/or functional fragment of a protein encoded by a DNA sequence as given in any of SEQ ID NOs 1 to 5,
- 20 (k) a nucleic acid which is degenerated to a nucleic acid as given in any of SEQ ID NOs 1 to 5, or which is degenerated to a nucleic acid as defined in any of (a) to (j) as a result of the genetic code,
- (l) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs 6 to 10, or which is diverging from a nucleic acid as defined in any of (a) to (j) as a result of differences in codon usage between organisms,
- 25 (m) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs 6 to 10, or which is diverging from a nucleic acid as defined in any of (a) to (j) as a result of differences between alleles, and
- (n) a nucleic acid as defined in any one of (a) to (m) characterized in that said nucleic acid is DNA, cDNA, genomic DNA or synthetic DNA.
- 30

Furthermore, one of the embodiments of this invention is a method for enhancing stress tolerance in a plant or in plants comprising expression of at least one of the nucleic acids as described above in cells, tissues or parts of said plant or plants. Also for those plants that already express a nucleic acid according to the invention, another embodiment of the present invention is a method for altering stress tolerance in said plants comprising

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altering the expression of a nucleic acid of the invention in cells, tissues or parts of said plants.

In an interesting embodiment the present invention relates to a method for induction of stress tolerance in an organism, for instance a plant, comprising the expression or altering the expression of a of a nucleic acid encoding a casein kinase alpha subunit, for instance a plant casein kinase alpha subunit in cells, tissues or parts of said organism. More preferably the present invention relates to a method for induction of osmotic, salt, Na<sup>+</sup> or Li<sup>+</sup> stress tolerance to an organism, e.g. a plant, yeast or bacteria, comprising the expression of a plant casein kinase  $\alpha$  subunit, such as a casein kinase  $\alpha$  subunit of sugar beet or a homologue or an orthologue thereof. In a preferred embodiment the present invention relates to a method for the induction of Na<sup>+</sup> tolerance in a plant, such as rice, comprising the expression of a casein kinase  $\alpha$  subunit of sugar beet.

In an interesting embodiment of the invention, said plant casein kinase  $\alpha$  subunit is represented by SEQ ID Nos 1 and 6.

According to another embodiment the invention relates to the use of a casein kinase to control the flowering process of plants. The present inventors surprisingly found that overexpression of a casein kinase  $\alpha$  subunit, such as the sugar beet casein kinase  $\alpha$  subunit identified in the present invention has an effect on the flowering process, independent from the light.

The invention thus relates to a method for controlling the process of flowering of a plant comprising the expression or altering the expression of a nucleic acid encoding a casein kinase alpha subunit, such as represented by SEQ ID NO 6 in cells, tissues or parts of said plant.

The invention thus also relates to the use of a nucleic acid encoding a casein kinase alpha subunit, such as represented by SEQ ID NO 6 for controlling the process of flowering of a plant.

Even so a preferred embodiment of the present invention relates to a method for enhancing stress tolerance in an organism, for instance in a plant, comprising the expression or altering the expression of a nucleic acid encoding a dihydroorotase in cells, tissues or parts of said organism or said plant.

More preferably the present invention relates to a method for induction of osmotic, salt, Na<sup>+</sup> or Li<sup>+</sup> stress tolerance in an organism, e.g. a plant, yeast or bacteria, comprising the expression of a plant dihydroorotase, such as dihydroorotase of sugar beet or a homologue or an orthologue or a paralogue thereof. In a preferred embodiment, the present invention relates to a method for the induction of Na<sup>+</sup> tolerance to a plant, such as rice, comprising the expression of the dihydroorotase of sugar beet. In an interesting

embodiment of the invention, said dihydroorotase is represented by SEQ ID Nos 2 and 7.

In another embodiment the present invention relates to a method for induction of stress tolerance in an organism, for instance in a plant, comprising the expression or altering the expression of a nucleic acid encoding a translation initiation factor 1A in cells, tissues or parts of said organism. More preferably the present invention relates to a method for induction of osmotic, salt, Na<sup>+</sup> or Li<sup>+</sup> stress tolerance in an organism, e.g. a plant, yeast or bacteria, comprising the expression of a plant translation initiation factor 1A, such as a translation initiation factor 1A of sugar beet or a homologue, an orthologue or a paralogue thereof. In a preferred embodiment of the present invention relates to a method for the induction of Na<sup>+</sup> tolerance in a plant, such as rice, comprising the expression of a translation initiation factor 1A of sugar beet. In an interesting embodiment of the invention, said translation initiation factor 1A is represented by SEQ ID Nos 3 and 8.

In another embodiment the present invention relates to a method for enhancing stress tolerance in a plant comprising the expression of or altering the expression of a nucleic acid as represented by SEQ ID NO 4 or 5, or a homologue, an orthologue or a paralogue thereof.

The present invention also relates to an isolated nucleic acid encoding a protein or an immunologically active and/or functional fragment of such a protein selected from the group consisting of:

- (a) a nucleic acid comprising a DNA sequence as given in any of SEQ ID NOs 1 to 5 or the complement thereof,
- (b) a nucleic acid comprising the RNA sequences corresponding to any of SEQ ID NOs 1 to 5 or the complement thereof,
- (c) a nucleic acid specifically hybridizing to the nucleotide sequence as defined in (a) or (b) under high stringency conditions,
- (d) a nucleic acid encoding a protein with an amino acid sequence which is at least 93%, preferably at least 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 6,
- (e) a nucleic acid encoding a protein with an amino acid sequence which is at least 80% preferably at least 82%, 85% 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 7,
- (f) a nucleic acid encoding a protein with an amino acid sequence which is at least 89%, preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 8,

- (g) a nucleic acid encoding a protein with an amino acid sequence which is at least 75% preferably at least 78%, 80%, 85%, 87%, 89%, 91%, 93%, 96%, 95%, 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 9,
- 5 (h) a nucleic acid encoding at least part of a protein with an amino acid sequence which is at least 65 % preferably at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence as given in SEQ ID NO 10,
- (i) a nucleic acid encoding a protein comprising the amino acid sequence as given in any of SEQ ID NOs 6-10,
- 10 (j) a nucleic acid encoding an immunologically active and/or functional fragment of a protein encoded by a DNA sequence as given in any of SEQ ID NOs 1 to 5,
- (k) a nucleic acid which is degenerated to a nucleic acid as given in any of SEQ ID NOs 1 to 5, or which is degenerated to a nucleic acid as defined in any of (a) to (j) as a result of the genetic code,
- 15 (l) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs 6 to 10, or which is diverging from a nucleic acid as defined in any of (a) to (j) as a result of differences in codon usage between organisms,
- (m) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs 6 to 10, or which is diverging from a nucleic acid as defined in any of (a) to (j) as a result of differences between alleles, and
- 20 (n) a nucleic acid as defined in any one of (a) to (m) characterized in that said nucleic acid is DNA, cDNA, genomic DNA or synthetic DNA.

The clone 154 (SEQ ID NO 1) was chosen for further characterization because it has homologues in yeast. Many information is available about CK2 in yeast and other organisms e.g. mutants of the yeast CK2 subunits.

- 25 In order to confirm the presence of *BvCKA2* in the sugar beet genome and to estimate the number of genes encoding the CK2 catalytic subunits in this plant species, the inventors performed a Southern blot analysis. As described in example 8 we first hybridized the genomic sugar beet genomic DNA using a fragment including the ORF of *BvCKA2* (Figure 2A). The presence of several hybridization fragments in all lanes independent of the
- 30 restriction endonucleases used to digest the genomic DNA, suggest that *CKA2* is a member of a multicopy gene family in sugar beet. The hybridization probe we used may recognize all the members of the *CK2* family, including genes coding for different isoforms of the catalytic subunit. When a more specific probe was used for hybridization, only two
- 35 bands in the *Bam* HI and *Hind* III digest, and one band in the *Eco* RI digest could be detected (Figure 2B). This may indicate the presence of two very closely related genes coding for CK2 catalytic subunits in sugar beet.

To confirm the functionality of the *BvCKA2* gene to confer stress tolerance to yeast, the complementation of the yeast *CK2* mutation was demonstrated (example 6).

In the yeast *Saccharomyces cerevisiae*, CK2 is essential for growth and there are two redundant genes, *CKA1* and *CKA2*, encoding two related catalytic subunits (Padmanabha *et al.* 1990). YDH8 is a *cka1/cka2* double mutant yeast strain that carries a plasmid with a thermosensitive *cka2* subunit (pDH8). To determine whether *BvCKA2* could suppress the thermosensitive phenotype of YDH8 strain, we transformed YDH8 cells with the plasmid pYPGE15+*BvCKA2*. While the YDH8 strain could only grow at the permissive temperature of 25°C, YDH8 overexpressing *BvCKA2* was able to grow at 25° and at 37°C (Figure 3).

*BvCKA2* could also complement other phenotypic characteristics of the *cka1,2* mutant strain such as flocculation (data not shown). Finally, when the pDH8 plasmid was removed and the cells only expressed *BvCKA2*, the plant enzyme could support yeast growth at 25° and 37°C (data not shown). These results clearly suggest that *BvCKA2* can functionally replace the yeast catalytic subunit of CK2 and this may suggest that CK2 is regulating related processes in both organisms

According to these aspects of the invention, that illustrate the cloning of the *BvCKA2* gene, the present invention refers to a nucleic acid molecule of at least 15 nucleotides in length specifically hybridizing with a nucleic acid of the invention.

In an a related preferred embodiment, the present invention also refers to nucleic acid molecule of at least 15 nucleotides in length specifically amplifying a nucleic acid of the invention.

Another preferred embodiment of the present invention is a vector comprising a nucleic acid sequence as defined above, such as an expression vector wherein the nucleic acid sequence is operably linked to one or more control sequences allowing the expression of said sequence in prokaryotic and/or eukaryotic host cells.

A further related embodiment of the present invention is a host cell containing a nucleic acid molecule as defined above or a vector as described above, such host cell for example being a bacterial, insect, fungal, plant or animal cell.

The functionality of the *BvCKA2* gene was also confirmed by the demonstration of growth of a Na<sup>+</sup> sensitive yeast strain JM26 (example 2) in media with NaCl as described in example 7.

This yeast mutant, defective in the non-essential  $\beta$  regulatory subunit of CK2, displays a phenotype of hypersensitivity to Na<sup>+</sup> and Li<sup>+</sup> (Bidwai *et al.* 1995, Nadal *et al.* 1999b). Overexpression of both the sugar beet catalytic alpha 2 subunit (*BvCKA2*) as well as one of the yeast catalytic subunits (*ScCKA2*), that was cloned as described in example 5, increased the Na<sup>+</sup> tolerance of the JM26 yeast cells (Figure 4). This indicates a specific

effect of CK2 on Na<sup>+</sup> tolerance. The increased Na<sup>+</sup> tolerance of yeast cells overexpressing *BvCKA2* could also be demonstrated in liquid cultures.

The above mentioned functional effects of overexpression of the sugar beet genes can possibly also be obtained by applying the isolated polypeptides produced by those genes or produced in a synthetic way.

Therefore, the present invention also relates to an isolated polypeptide encodable by a nucleic acid of the present invention as described above, or a homologue or a derivative thereof, or an immunologically active and/or functional fragment thereof, this polypeptide preferably having an amino acid sequence as given in any of SEQ ID NO 6-10, or a homologue or a derivative thereof, or an immunologically active and/or functional fragment thereof.

Also a related preferred embodiment of the present invention is a method for producing a polypeptide as mentioned above comprising culturing a host cell as mentioned above, under conditions allowing the expression of the polypeptide and recovering the produced polypeptide from the culture.

Also the functional effects that were seen upon expression of the sugar beet genes could be influenced by proteins that bind to the polypeptides produced by those genes. Therefore, in yet another preferred embodiment, the present invention relates to an antibody specifically recognizing a polypeptide as mentioned above or a specific epitope of said polypeptide.

In order to investigate whether the mechanism of salt tolerance conferred by *BvCKA2* was due to the regulation of ion homeostasis, the intracellular levels of Na<sup>+</sup> and K<sup>+</sup> in cells growing in the presence of these ions (Table 1) was determined as described in example 7. It was determined that expression of *BvCKA2* did not significantly change the Na<sup>+</sup> and K<sup>+</sup> content in yeast cells.

***Table 1: Potassium and sodium content (mM) of JM26 cells overexpressing CKA2. Cells were grown overnight in the presence of 75 mM NaCl. Results are the mean of three independent experiments  $\pm$  SD.***

	[K]	[Na]
PYPGE15	57 $\pm$ 11	136 $\pm$ 9
PYPGE15+BvCKA2	56 $\pm$ 3	146 $\pm$ 9

In yeast, it has been shown that mutants in the  $\beta$  regulatory subunit of the CK2 (*CKB1*) are highly sensitive to Na<sup>+</sup> and Li<sup>+</sup> (Bidwai *et al.* 1995, Nadal *et al.* 1999a). An aspect of the present invention is that overexpression of not only the yeast but also the plant CK2  $\alpha$

catalytic subunit increases yeast tolerance to  $\text{Na}^+$ . Since not only reduction of CK2 activity increases sensitivity to NaCl (*ckb1* yeast mutants) but also increase in CK2 activity improves salt tolerance, it is possible that CK2 is an important determinant of salt tolerance in yeast. However, the mechanism by which CK2 may regulate yeast salt tolerance is not known. Recently published data suggest that CK2 regulated the transcription of the ENA1 ATPase (Teney and Glover, 1999), the main determinant for  $\text{Na}^+$  efflux in yeast. One of the aspects of the invention teaches away from this, since it was demonstrated that the salt tolerance conferred by BvCKA2 is not related to the regulation of the  $\text{Na}^+$  homeostasis within the cells. Furthermore it was shown that overexpression of BvCKA2 improved the salt tolerance of the JM26 yeast mutant lacking the two major transport systems involved in the  $\text{Na}^+$  efflux (the ENA1-4 ATPase and the  $\text{Na}^+/\text{H}^+$  antiporter NHA1). In addition, measurements of intracellular  $\text{Na}^+$  and  $\text{K}^+$  did not show any significant difference between controls and cells overexpressing CK2 (Table 1). Yeast cells lacking the vacuolar  $\text{Na}^+/\text{H}^+$  antiporter NHX also showed improved growth in media with NaCl when they overexpressed CK2 (data not shown) ruling out the possibility that CK2 alters the cytoplasmic levels of  $\text{Na}^+$  through vacuolar sequestration. According to this, Nadal *et al.* (1999b) showed that the salt sensitivity of the yeast *ckb1* mutant was not due to defects in the fluxes of sodium. These authors postulated that CK2 might reduce, by phosphorylation, the  $\text{Na}^+$  sensitivity of an important component of the cellular machinery that is salt sensitive. It is difficult to find such putative targets of salt toxicity since many substrates including transcription factors, protein kinases and topoisomerases have been found to possess putative CK2 phosphorylation sites (Grein *et al.* 1999).

In order to confirm that BvCKA2 participates in the response of sugar beet plants to salt stress the accumulation of BvCKA2 mRNA in response to various exposure times to NaCl was analyzed. RNA gel blot using a CK2 specific probe showed only one band that corresponded to the size of the BvCKA2 cDNA (1.5 Kb). As shown in figure 5 the BvCKA2 mRNA accumulated with time upon NaCl treatment, and reached a maximum at 24 hours. The increase was about 3-fold as compared to control plants. It is interesting to note that the sugar beet cDNA library used to search for genes that are involved in stress tolerance was also obtained from plants treated for 24 hours with NaCl.

In order to confirm that BveIF-1A participates in the response of yeast cells to salt stress the incorporation of phenylalanine in proteins was measured. The inventors observed that under salt stress conditions the incorporation of phenylalanine in yeast cells transformed with the cDNA encoding BveIF-1A was much higher than in cells transformed with the empty vector (example 12). These results demonstrate that eIF-1a is directly involved in the response to stress tolerance. Furthermore these results show that overexpression of this cDNA (according to SEQ ID Nr. 3) can improve translation under salt stress condition.

These new findings are very interesting since this is the first evidence showing that a translation initiation factor improves stress tolerance.

The sugar beet genes of the present invention were shown to confer stress tolerance to a heterologous yeast when transfected herein.

5 Furthermore, the sugar beet genes of the present invention were shown to confer stress tolerance to a plant when transfected herein (see example 10).

A further and agronomically interesting application of these genes, is to transfect them into a crop plant in order to render this crop more tolerant to unfavourable growth conditions. Rice plants can be transfected with at least one of the sugar beet genes of the present invention (example 9), which can confer salt tolerance to the rice plants. This application  
10 offers a solution to the reduced yield of rice in heavily irrigated lands, caused by the deposition and accumulation of salts from the irrigation water in the soil.

According to the above mentioned aspects of the invention, documenting the effect of the sugar beet gene in a physiologic response to a stress situation *in planta*, the present  
15 invention also relates to a method for the production of transgenic plants, plant cells or plant tissues comprising the introduction of a nucleic acid molecule as defined above in an expressible format or a vector as defined above in said plant, plant cell or plant tissue.

Also within the scope of the present invention is a method for the production of altered plants, plant cells or plant tissues comprising the introduction of a polypeptide of the  
20 present invention directly into a cell, a tissue or an organ of said plant.

In a further embodiment the present invention relates to a method for enhancing stress tolerance in a plant cell, tissue or plant comprising the introduction of any nucleic acid as mentioned above into said plant cell, tissue or organ of said plant.

Furthermore, the present invention provides in a preferred embodiment for a method to  
25 effect the expression of a polypeptide as mentioned above comprising the introduction of a nucleic acid molecule of the present invention, optionally operably linked to one or more control sequences or a vector as defined above stably into the genome of a plant cell. Even so in a related embodiment, the invention provides for a method as described here above, further comprising regenerating a plant from said plant cell.

30 In a further embodiment, the invention here disclosed, relates to a transgenic plant cell comprising a nucleic acid sequence of the invention which is operable linked to regulatory elements allowing transcription and/or expression of said nucleic acid in plant cells or a transgenic plant cell obtainable by a method as described above. Furthermore, this transgenic plant cell can have said nucleic acid of the invention stably integrated into its

35 genome. Also in the scope of the present invention is a transgenic plant or plant tissue comprising plant cells as described above, and furthermore this transgenic plant can display increased tolerance to stress, preferably osmotic stress such as salt, Na<sup>+</sup>, Li<sup>+</sup>,



drought, cold or freezing stress or oxidative stress, compared to the corresponding wild type plant. A related embodiment of the present invention is a harvestable part of such a plant which can be selected from the group consisting of seeds, leaves, fruits, stem cultures, rhizomes and bulbs or the progeny derived from any of the plants or plant parts as described above.

Because it was demonstrated that the sugar beet genes of the present invention are not functionally restricted to their homologous background, the scope of the present invention also refers to a method for altering stress tolerance in (a) organism(s) comprising the expression or altering the expression of a nucleic acid of the invention in cells, tissues or parts of said organism(s).

As known by the person skilled in the art, many genes involved in salt stress are also involved in responses to other stress situations. Accordingly, the present invention also relates to above mentioned methods for producing transgenic cells, plants or other organisms, wherein said stress can be osmotic stress, salt stress, drought stress, freezing stress or cold stress or oxidative stress.

In most practical applications of the present invention, the novel technology will be used to create a beneficial effect for the transformed organism. Therefore in a most preferred embodiment, the present invention relates to a method according to any of the methods as mentioned above, said method leading to an increase in yield and even so to a method wherein said expression of said nucleic acid occurs under the control of a promoter. Said promoter can be a constitutive or inducible promoter. In cases where cell-specific, tissue-specific or organ-specific expression of genes is envisaged, a cell-specific, tissue-specific or organ-specific promoter is used. An exhaustive but non-limiting list of examples of promoters that can be used in the methods of the invention is provided in Table 4.

As for yeast, many interacting partners for the plant CK2 have been postulated, including transcription factors, such as CCA1 involved in regulation of the circadian clock (Sugano *et al.* 1999), or GBF1 that regulates the expression of light inducible genes (Donald and Cashmore 1990). Interestingly, two protein kinases induced by salt stress, ATPK19 and ATPK6, also contain putative CK2 phosphorylation sites (Mizoguchi *et al.* 1995).

Accordingly, a preferred embodiment of the present invention is a method for identifying and obtaining proteins interacting with a polypeptide of the present invention comprising a screening assay wherein a polypeptide of the present invention is used. This method could for example comprise a two-hybrid screening assay wherein a polypeptide of the present invention as a bait and a cDNA library as prey are used.

Also a method for modulating the interaction between a polypeptide of the present invention and interacting protein partners obtainable by a method as described above is in the scope of the present invention. Furthermore, the present invention embodies a method

for identifying and obtaining compounds interacting with a polypeptide of the present invention comprising the steps of:

- (a) providing a two-hybrid system wherein a polypeptide of the present invention and an interacting protein partner obtainable by a method as described above are expressed,
- (b) interacting said compound with the complex formed by the expressed polypeptides as defined in a), and,
- (c) performing measurement of interaction of said compound with said polypeptide or the complex formed by the expressed polypeptides as defined in (a).

Even so, the present invention embodies a method for identifying compounds or mixtures of compounds which specifically bind to a polypeptide of the present invention, comprising:

- (a) combining a polypeptide of the present invention with said compound or mixtures of compounds under conditions suitable to allow complex formation, and,
- (b) detecting complex formation, wherein the presence of a complex identifies a compound or mixture of compounds which specifically binds said polypeptide.

Because these interaction partners of the polypeptide of the present invention can cooperate in the functionality of these polypeptides, the present invention also embodies the use of a compound or mixture of compounds identified by means of a method as described above as a factor that enhances stress tolerance in (a) organism(s).

Accordingly, the present invention embodies the use of a nucleic acid molecule of the invention as defined above, a vector of the invention, a polypeptide of the invention for increasing yield or for stimulating plant growth. In particular the present invention offers the opportunity to increase the yield of any harvestable part of a plant, such as root, leaf, seeds etc.

It is important for the agronomic success of a crop to be able to cope with stress situations. Therefore it could be useful to use the genes or the polypeptides of the present invention to screen important crops for the presence of stress tolerance genes. Accordingly the present invention relates to a diagnostic composition comprising at least a nucleic acid of the invention, a vector of the invention, a polypeptide of the invention or an antibody of the invention.

It is described that in normal plant growing conditions there is a typically low concentration of less than 1 mM Na<sup>+</sup>. Therefore the present invention offers the possibility to use a plant obtainable by the method as defined above or the plant of the invention for culturing on soil with a salt content of more than 1 mM ions. In most experiments it was shown that salt tolerant plants were able to grow on in conditions with about 40 mM to about 400 mM Na<sup>+</sup>.

## DEFINITIONS AND ELABORATIONS TO THE EMBODIMENTS

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

The terms "protein(s)", "peptide(s)" or "oligopeptide(s)", when used herein refer to amino acids in a polymeric form of any length. Said terms also include known amino acid modifications such as disulphide bond formation, cysteinylation, oxidation, glutathionylation, methylation, acetylation, farnesylation, biotinylation, stearylation, formylation, lipoic acid addition, phosphorylation, sulphation, ubiquitination, myristoylation, palmitoylation, geranylgeranylation, cyclization (e.g. pyroglutamic acid formation), oxidation, deamidation, dehydration, glycosylation (e.g. pentoses, hexosamines, N-acetylhexosamines, deoxyhexoses, hexoses, sialic acid etc.), acylation and radiolabels (e.g. <sup>125</sup>I, <sup>131</sup>I, <sup>35</sup>S, <sup>14</sup>C, <sup>32</sup>P, <sup>33</sup>P, <sup>3</sup>H) as well as non-naturally occurring amino acid residues, L-amino acid residues and D-amino acid residues.

"Homologues" or "Homologs" of a protein of the invention are those peptides, oligopeptides, polypeptides, proteins and enzymes which contain amino acid substitutions, deletions and/or additions relative to the said protein with respect to which they are a homologue without altering one or more of its functional properties, in particular without reducing the activity of the resulting product. For example, a homologue of said protein will consist of a bioactive amino acid sequence variant of said protein. To produce such homologues, amino acids present in the said protein can be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break  $\alpha$ -helical structures or  $\beta$ -sheet structures, and so on. An overview of physical and chemical properties of amino acids is given in Table 2.

**Table 2: Properties of naturally occurring amino acids.**

Charge properties / hydrophobicity	Side group	Amino Acid
nonpolar hydrophobic	aliphatic aliphatic, S-containing aromatic imino	ala, ile, leu, val met phe, trp pro
polar uncharged	aliphatic amide aromatic hydroxyl sulfhydryl	gly asn, gln tyr ser, thr cys
positively charged	basic	arg, his, lys
negatively charged	acidic	asp, gly

Two special forms of homology, orthologous and paralogous, are evolutionary concepts used to describe ancestral relationships of genes. The term "paralogous" relates to gene-  
 5 duplications within the genome of a species leading to paralogous genes. The term "orthologous" relates to homologous genes in different organisms due to ancestral relationship. The present invention thus also relates to homologues, paralogues and orthologues of the genes and proteins of the invention. The paralogues or orthologues of the genes and proteins of the invention may have a lesser percentage of sequence  
 10 identity with the sequences or proteins of the invention than the strictly interpreted "homologues" as defined earlier.

Substitutional variants of a protein of the invention are those in which at least one residue in said protein amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered  
 15 depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Insertional amino acid sequence variants of a protein of the invention are those in which  
 20 one or more amino acid residues are introduced into a predetermined site in said protein. Insertions can comprise amino-terminal and/or carboxy-terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 10 residues. Examples of amino- or carboxy-terminal fusion proteins or  
 25 peptides include the binding domain or activation domain of a transcriptional activator as used in the yeast two-hybrid system, phage coat proteins, (histidine)<sub>6</sub>-tag, glutathione S-

transferase, protein A, maltose-binding protein, dihydrofolate reductase, Tag•100 epitope (EETARFQPGYRS), c-myc epitope (EQKLISEEDL), FLAG®-epitope (DYKDDDDK), lacZ, CMP (calmodulin-binding peptide), HA epitope (YPYDVPDYA), protein C epitope (EDQVDPRLIDGK) and VSV epitope (YTDIEMNRLGK).

- 5 Deletional variants of a protein of the invention are characterised by the removal of one or more amino acids from the amino acid sequence of said protein.

Amino acid variants of a protein of the invention may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to  
10 produce variant proteins, which manifest as substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis, T7-Gen in vitro mutagenesis kit (USB, Cleveland, OH), QuickChange Site Directed mutagenesis kit (Stratagene, San Diego, CA), PCR-mediated  
15 site-directed mutagenesis or other site-directed mutagenesis protocols. Another alternative to manipulate DNA sequences to produce variant proteins, which manifest as substitutional, insertional or deletional variants comprises targeted *in vivo* gene modification which can be achieved by chimeric RNA/DNA oligonucleotides as described by e.g. (Palmgren 1997; Yoon *et al.* 1996).

- 20 "Derivatives" of a protein of the invention are those peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise additional naturally-occurring, altered glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of said polypeptide. Alternatively or in addition, a derivative may comprise one or more non-amino acid substituents compared to  
25 the amino acid sequence of a naturally-occurring form of said polypeptide, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound thereto to facilitate its detection. A derivative of a protein retains the biological or enzymatical activity of the protein where it is derived from.

- 30 With "immunologically active" is meant that a molecule or specific fragments thereof such as epitopes or haptens are recognised by, i.e. bind to antibodies.

In the context of the current invention are also included homologous, derivatives and/or immunologically active fragments of any of the inventive sugar beet polypeptides or homologue, derivative or fragment thereof as defined *supra*.

- 35 "Antibodies" include monoclonal, polyclonal, synthetic or heavy chain camel antibodies as well as fragments of antibodies such as Fab, Fv or scFv fragments. Monoclonal antibodies can be prepared by the techniques as described previously e.g. (Liddle & Cryer 1991)

which comprise the fusion of mouse myeloma cells to spleen cells derived from immunised animals. Furthermore, antibodies or fragments thereof to a molecule or fragments thereof can be obtained by using methods as described in e.g. (Harlow & Lane 1988). In the case of antibodies directed against small peptides such as fragments of a protein of the invention, said peptides are generally coupled to a carrier protein before immunisation of animals. Such protein carriers include keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin and Tetanus toxoid. The carrier protein enhances the immune response of the animal and provides epitopes for T-cell receptor binding sites. The term "antibodies" furthermore includes derivatives thereof such as labelled antibodies.

Antibody labels include alkaline phosphatase, PKH2, PKH26, PKH67, fluorescein (FITC), Hoechst 33258, R-phycoerythrin (PE), rhodamine (TRITC), Quantum Red, Texas Red, Cy3, biotin, agarose, peroxidase, gold spheres and radiolabels (e.g.  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^3\text{H}$ ). Tools in molecular biology relying on antibodies against a protein include protein gel blot analysis, screening of expression libraries allowing gene identification, protein quantitative methods including ELISA and RIA, immunoaffinity purification of proteins, immunoprecipitation of proteins e.g. (Magyar *et al.* 1997) and immunolocalization. Other uses of antibodies and especially of peptide antibodies include the study of proteolytic processing (Loffler *et al.* 1994; Woulfe *et al.* 1994), determination of protein active sites (Lerner 1982), the study of precursor and post-translational processing (Baron & Baltimore 1982; Lerner *et al.* 1981; Semler *et al.* 1982), identification of protein domains involved in protein-protein interactions (Murakami *et al.* 1992) and the study of exon usage in gene expression (Tamura *et al.* 1991).

In the scope of the current invention are also antibodies recognising the inventive sugar beet polypeptides, derivative or fragment thereof as defined *supra*.

The terms "gene(s)", "polynucleotide(s)", "nucleic acid sequence(s)", "nucleotide sequence(s)", "DNA sequence(s)" or "nucleic acid molecule(s)", when used herein refer to nucleotides, either ribonucleotides or deoxyribonucleotides or a combination of both, in a polymeric form of any length. Said terms furthermore include double-stranded and single-stranded DNA and RNA. Said terms also include known nucleotide modifications such as methylation, cyclization and 'caps' and substitution of one or more of the naturally occurring nucleotides with an analogue such as inosine. Modifications of nucleotides include the addition of acridine, amine, biotin, cascade blue, cholesterol, Cy3®, Cy5®, Cy5.5® Dabcyl, digoxigenin, dinitrophenyl, Edans, 6-FAM, fluorescein, 3'-glyceryl, HEX, IRD-700, IRD-800, JOE, phosphate psoralen, rhodamine, ROX, thiol (SH), spacers, TAMRA, TET, AMCA-S®, SE, BODIPY®, Marina Blue®, Pacific Blue®, Oregon Green®, Rhodamine Green®, Rhodamine Red®, Rhodol Green® and Texas Red®. Polynucleotide backbone modifications include methylphosphonate, 2'-OMe-methylphosphonate RNA,

phosphorothiorate, RNA, 2'-OMeRNA. Base modifications include 2-amino-dA, 2-aminopurine, 3'-(ddA), 3'dA(cordycepin), 7-deaza-dA, 8-Br-dA, 8-oxo-dA, N<sup>6</sup>-Me-dA, abasic site (dSpacer), biotin dT, 2'-OMe-5Me-C, 2'-OMe-propynyl-C, 3'-(5-Me-dC), 3'-(ddC), 5-Br-dC, 5-I-dC, 5-Me-dC, 5-F-dC, carboxy-dT, convertible dA, convertible dC, convertible dG, convertible dT, convertible dU, 7-deaza-dG, 8-Br-dG, 8-oxo-dG, O<sup>6</sup>-Me-dG, S6-DNP-dG, 4-methyl-indole, 5-nitroindole, 2'-OMe-inosine, 2'-dl, O<sup>6</sup>-phenyl-dl, 4-methyl-indole, 2'-deoxynebularine, 5-nitroindole, 2-aminopurine, dP(purine analogue), dK(pyrimidine analogue), 3-nitropyrrole, 2-thio-dT, 4-thio-dT, biotin-dT, carboxy-dT, O<sup>4</sup>-Me-dT, O<sup>4</sup>-triazol dT, 2'-OMe-propynyl-U, 5-Br-dU, 2'-dU, 5-F-dU, 5-I-dU, O<sup>4</sup>-triazol dU and radiolabels (e.g. <sup>125</sup>I, <sup>131</sup>I, <sup>35</sup>S, <sup>14</sup>C, <sup>32</sup>P, <sup>33</sup>P, <sup>3</sup>H). Said terms also encompass peptide nucleic acids (PNAs), a DNA analogue in which the backbone is a pseudopeptide consisting of N-(2-aminoethyl)-glycine units rather than a sugar. PNAs mimic the behaviour of DNA and bind complementary nucleic acid strands. The neutral backbone of PNA results in stronger binding and greater specificity than normally achieved. In addition, the unique chemical, physical and biological properties of PNA have been exploited to produce powerful biomolecular tools, antisense and antigene agents, molecular probes and biosensors. With "recombinant DNA molecule" or "chimeric gene" is meant a hybrid DNA produced by joining pieces of DNA from different sources. With "heterologous nucleotide sequence" is intended a sequence that is not naturally occurring with the promoter sequence. While this nucleotide sequence is heterologous to the promoter sequence, it may be homologous, or native, or heterologous, or foreign, to the plant host. "Sense strand" refers to the strand of a double-stranded DNA molecule that is homologous to a mRNA transcript thereof. The "anti-sense strand" contains an inverted sequence, which is complementary to that of the "sense strand".

A "coding sequence" or "open reading frame" or "ORF" is defined as a nucleotide sequence that can be transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences, i.e. when said coding sequence or ORF is present in an expressible format. Said coding sequence or ORF is bounded by a 5' translation start codon and a 3' translation stop codon. A coding sequence or ORF can include, but is not limited to RNA, mRNA, cDNA, recombinant nucleotide sequences, synthetically manufactured nucleotide sequences or genomic DNA. Said coding sequence or ORF can be interrupted by intervening nucleic acid sequences. Genes and coding sequences essentially encoding the same protein but isolated from different sources can consist of substantially divergent nucleic acid sequences. Reciprocally, substantially divergent nucleic acid sequences can be designed to effect expression of essentially the same protein. Said nucleic acid sequences are the result of e.g. the existence of different alleles of a given gene, or of the degeneracy of the genetic

code or of differences in codon usage. Thus, as indicated in Table 3, amino acids such as methionine and tryptophan are encoded by a single codon whereas other amino acids such as arginine, leucine and serine can each be translated from up to six different codons. Differences in preferred codon usage are illustrated below for *Agrobacterium tumefaciens* (a bacterium), *Arabidopsis thaliana*, *M. sativa* (two dicotyledonous plants) and *Oryza sativa* (a monocotyledonous plant). These examples were extracted from (<http://www.kazusa.or.jp/codon>). To give one example, the codon GGC (for glycine) is the most frequently used codon in *Agrobacterium tumefaciens* (36.2 ‰), is the second most frequently used codon in *Oryza sativa* but is used at much lower frequencies in *Arabidopsis thaliana* and *M. sativa* (9 ‰ and 8.4 ‰, respectively). Of the four possible codons encoding glycine (see Table 3), said GGC codon is most preferably used in *Agrobacterium tumefaciens* and *Oryza sativa*. However, in *Arabidopsis thaliana* this is the GGA (and GGU) codon whereas in *M. sativa* this is the GGU (and GGA) codon. Allelic variants are further defined as to comprise single nucleotide polymorphisms (SNPs) as well as small insertion/deletion polymorphisms (INDELs; the size of INDELs is usually less than 100 bp). SNPs and INDELs form the largest set of sequence variants in naturally occurring polymorphic strains of most organisms. They are helpful in mapping genes and discovery of genes and gene functions. They are furthermore helpful in identification of genetic loci, e.g. plant genes, involved in determining processes such as growth rate, plant size and plant yield, plant vigor, disease resistance, stress tolerance etc.



Table 3. Degeneracy of the genetic code.

Amino Acid	Three-letter code	One-letter code	Possible codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Asparagine	Asn	N	AAC	AAU				
Aspartic Acid	Asp	D	GAC	GAU				
Cysteine	Cys	C	UGC	UGU				
Glutamic Acid	Glu	E	GAA	GAG				
Glutamine	Gln	Q	CAA	CAG				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Lysine	Lys	K	AAA	AAG				
Methionine	Met	M	AUG					
Phenylalanine	Phe	F	UUC	UUU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				
Valine	Val	V	GUA	GUC	GUG	GUU		
Possible "STOP" codons								
			UAA	UAG	UGA			

- 5 Many techniques are nowadays available to identify SNPs and/or INDELs including (i) PCR followed by denaturing high performance liquid chromatography (DHPLC; e.g. Cho *et al.* 1999); (ii) constant denaturant capillary electrophoresis (CDCE) combined with high-fidelity PCR (e.g. Li-Sucholeiki *et al.* 1999); (iii) denaturing gradient gel electrophoresis (e.g. Fischer and Lerman 1983); (iv) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; e.g. Ross *et al.* 2000); (v) real-time fluorescence monitoring PCR assays (e.g. Tapp *et al.* 2000); (vi) Acrydite<sup>TM</sup> gel technology (e.g. Kenney *et al.* 1998); (vii) cycle dideoxy fingerprinting (CddF; e.g. Langemeier *et al.* 1994); (viii) single-strand conformation polymorphism (SSCP) analysis
- 10

(e.g. Vidal-Puig and Moller 1994) and (ix) mini-sequencing primer extension reaction (e.g. Syvanen 1999). The technique of 'Targeting Induced Local Lesions in Genomes' (TILLING; McCallum *et al.* 2000a,b), which is a variant of (i) *supra*, can also be applied to rapidly identify an altered gene in e.g. chemically mutagenized plant individuals showing interesting phenotypes.

"Hybridisation" is the process wherein substantially homologous complementary nucleotide sequences anneal to each other. The hybridisation process can occur entirely in solution, i.e. both complementary nucleic acids are in solution. Tools in molecular biology relying on such a process include the polymerase chain reaction (PCR; and all methods based thereon), subtractive hybridisation, random primer extension, nuclease S1 mapping, primer extension, reverse transcription, cDNA synthesis, differential display of RNAs, and DNA sequence determination. The hybridisation process can also occur with one of the complementary nucleic acids immobilised to a matrix such as magnetic beads, Sepharose beads or any other resin. Tools in molecular biology relying on such a process include the isolation of poly (A+) mRNA. The hybridisation process can furthermore occur with one of the complementary nucleic acids immobilised to a solid support such as a nitro-cellulose or nylon membrane or immobilised by e.g. photolithography to e.g. a siliceous glass support (the latter known as nucleic acid arrays or microarrays or as nucleic acid chips). Tools in molecular biology relying on such a process include RNA and DNA gel blot analysis, colony hybridisation, plaque hybridisation, in situ hybridisation and microarray hybridisation. In order to allow hybridisation to occur, the nucleic acid molecules are generally thermally or chemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridisation is influenced by conditions such as temperature, salt concentration and hybridisation buffer composition. High stringency conditions for hybridisation include high temperature and/or low salt concentration (salts include NaCl and Na<sub>3</sub>-citrate) and/or the inclusion of formamide in the hybridisation buffer and/or lowering the concentration of compounds such as SDS (detergent) in the hybridisation buffer and/or exclusion of compounds such as dextran sulphate or polyethylene glycol (promoting molecular crowding) from the hybridisation buffer. Conventional hybridisation conditions are described e.g. (Sambrook *et al.* 1989) but the skilled craftsman will appreciate that numerous different hybridisation conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid sequence. With specifically hybridising is meant hybridising under stringent conditions. Sufficiently low stringency hybridisation conditions are particularly preferred to isolate nucleic acids heterologous to the DNA sequences of the invention defined *supra*.

Elements contributing to said heterology include allelism, degeneration of the genetic code and differences in preferred codon usage as discussed *supra*.

Accordingly, the current invention is also related to the use of the inventive DNA sequences encoding the inventive sugar beet polypeptides, homologue, derivative and/or immunologically fragment thereof as defined higher in any method of hybridisation. The current invention furthermore also relates to DNA sequences hybridising to said inventive DNA sequences.

DNA sequences as defined in the current invention can be interrupted by intervening sequences. With "intervening sequences" is meant any nucleic acid sequence which disrupts a coding sequence comprising said inventive DNA sequence or which disrupts the expressible format of a DNA sequence comprising said inventive DNA sequence. Removal of the intervening sequence restores said coding sequence or said expressible format. Examples of intervening sequences include introns, mobilizable DNA sequences such as transposons and DNA tags such as e.g. a T-DNA. With "mobilizable DNA sequence" is meant any DNA sequence that can be mobilised as the result of a recombination event.

To effect expression of a protein in a cell, tissue or organ, preferably of plant origin, either the protein may be introduced directly to said cell, such as by microinjection or ballistic means or alternatively, an isolated nucleic acid molecule encoding said protein may be introduced into said cell, tissue or organ in an expressible format.

Preferably, the DNA sequence of the invention comprises a coding sequence or open reading frame (ORF) encoding the inventive sugar beet polypeptides or a homologue or derivative thereof or an immunologically active thereof as defined *supra*.

With "vector" or "vector sequence" is meant a DNA sequence, which can be introduced in an organism by transformation and can be stably maintained in said organism. Vector maintenance is possible in e.g. cultures of *Escherichia coli*, *Agrobacterium tumefaciens*, *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Other vectors such as phagemids and cosmid vectors can be maintained and multiplied in bacteria and/or viruses. Vector sequences generally comprise a set of unique sites recognised by restriction enzymes, the multiple cloning site (MCS), wherein one or more non-vector sequence(s) can be inserted.

With "non-vector sequence" is accordingly meant a DNA sequence which is integrated in one or more of the sites of the MCS comprised within a vector.

"Expression vectors" form a subset of vectors which, by virtue of comprising the appropriate regulatory sequences enabling the creation of an expressible format for the inserted non-vector sequence(s), thus allowing expression of the protein encoded by said non-vector sequence(s). Expression vectors are known in the art enabling protein- (gene-)

expression in organisms including bacteria (e.g. *Escherichia coli*), fungi (e.g. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*), insect cells (e.g. baculoviral expression vectors), animal cells (e.g. COS or CHO cells) and plant cells (e.g. potato virus X-based expression vectors, see e.g. Vance *et al.* 1998 - WO9844097).

- 5 The current invention clearly includes any vector or expression vector comprising a non-vector DNA sequence encoding the inventive sugar beet polypeptides, homologue, derivative and/or immunologically active fragment thereof as defined *supra*.

As an alternative to (expression) vector-mediated protein production in biological systems, chemical protein synthesis can be applied. Synthetic peptides can be manufactured in  
10 solution phase or in solid phase. Solid phase peptide synthesis (Merrifield, 1963) is, however, the most common way and involves the sequential addition of amino acids to create a linear peptide chain. Solid phase peptide synthesis includes cycles consisting of three steps: (i) immobilisation of the carboxy-terminal amino acid of the growing peptide chain to a solid support or resin; (ii) chain assembly, a process consisting of activation,  
15 coupling and deprotection of the amino acid to be added to the growing peptide chain; and (iii) cleavage involving removal of the completed peptide chain from the resin and removal of the protecting groups from the amino acid side chains. Common approaches in solid phase peptide synthesis include Fmoc/tBu (9-fluorenylmethyloxycarbonyl/t-butyl) and Boc (t-butyloxycarbonyl) as the amino-terminal protecting groups of amino acids. Amino acid  
20 side chain protecting groups include methyl (Me), formyl (CHO), ethyl (Et), acetyl (Ac), t-butyl (t-Bu), anisyl, benzyl (Bzl), trifluoroacetyl (Tfa), N-hydroxysuccinimide (ONSu, OSu), benzoyl (Bz), 4-methylbenzyl (Meb), thioanizyl, thiocresyl, benzyloxymethyl (Bom), 4-nitrophenyl (ONp), benzyloxycarbonyl (Z), 2-nitrobenzoyl (NBz), 2-nitrophenylsulphenyl (Nps), 4-toluenesulphonyl (Tosyl, Tos), pentafluorophenyl (Pfp), diphenylmethyl (Dpm), 2-  
25 chlorobenzyloxycarbonyl (Cl-Z), 2,4,5-trichlorophenyl, 2-bromobenzyloxycarbonyl (Br-Z), triphenylmethyl (Trityl, Trt), and 2,5,7,8-pentamethyl-chroman-6-sulphonyl (Pmc). During chain assembly, Fmoc or Boc are removed resulting in an activated amino-terminus of the amino acid residue bound to the growing chain. The carboxy-terminus of the incoming amino acid is activated by conversion into a highly reactive ester, e.g. by HBTU. With  
30 current technologies (e.g. PerSeptive Biosystems 9050 synthesizer, Applied Biosystems Model 431A Peptide Synthesizer), linear peptides of up to 50 residues can be manufactured. A number of guidelines is available to produce peptides that are suitable for use in biological systems including (i) limiting the use of difficult amino acids such as cys, met, trp (easily oxidised and/or degraded during peptide synthesis) or arg; (ii)  
35 minimize hydrophobic amino acids (can impair peptide solubility); and (iii) prevent an amino-terminal glutamic acid (can cyclize to pyroglutamate).

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein, either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (mitogens, anoxia, hypoxia, temperature, salt, light, dehydration, etc) or a chemical compound such as IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) or such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin), hormone (e.g. gibberellin, auxin, cytokinin, glucocorticoid, brassinosteroid, ethylene, abscisic acid etc), hormone analogue (iodoacetic acid (IAA), 2,4-D, etc), metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more post-translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term "expressible format".

Preferably, expression of a protein in a specific cell, tissue, or organ, preferably of plant origin, is effected by introducing and expressing an isolated nucleic acid molecule encoding said protein, such as a cDNA molecule, genomic gene, synthetic oligonucleotide molecule, mRNA molecule or open reading frame, to said cell, tissue or organ, wherein said nucleic acid molecule is placed operably in connection with suitable regulatory sequences including a promoter, preferably a plant-expressible promoter, and a terminator sequence.

"Regulatory sequence" refers to control DNA sequences, which are necessary to affect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, control sequences generally include promoters, ribosomal binding sites, and terminators. In eukaryotes generally control sequences include promoters, terminators and enhancers or silencers. The term "control sequence" is intended to include, at a minimum, all components the presence of which are necessary for expression, and may also include additional advantageous components which determines when, how much and where a specific gene is expressed.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner.

Regulatory sequences herein also refer to any of the group comprising a promoter, enhancer, silencer, intron sequence, 3'UTR and/or 5'UTR region, protein and /or RNA stabilizing elements.

5 The term "promoter" also includes the transcriptional regulatory sequences of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or a -10 box transcriptional regulatory sequences.

The term "promoter" is also used to describe a synthetic or fusion molecule or derivative, which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

10 Promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. Such regulatory elements may be placed adjacent to a heterologous promoter sequence to drive expression of a nucleic acid molecule in response to e.g. copper, glucocorticoids, 15 dexamethasone, tetracycline, gibberellin, cAMP, abscisic acid, auxin, wounding, ethylene, jasmonate or salicylic acid or to confer expression of a nucleic acid molecule to specific cells, tissues or organs such as meristems, leaves, roots, embryo, flowers, seeds or fruits. In the context of the present invention, the promoter preferably is a plant-expressible promoter sequence. Promoters, however, that also function or solely function in non-plant 20 cells such as bacteria, yeast cells, insect cells and animal cells are not excluded from the invention. By "plant-expressible" is meant that the promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell, tissue or organ, preferably a monocotyledonous or dicotyledonous plant cell, tissue, or organ.

25 The terms "plant-operable" and "operable in a plant" when used herein, in respect of a promoter sequence, shall be taken to be equivalent to a plant-expressible promoter sequence.

In the present context, a "regulated promoter" or "regulatable promoter sequence" is a promoter that is capable of conferring expression on a structural gene in a particular cell, 30 tissue, or organ or group of cells, tissues or organs of a plant, optionally under specific conditions, however does generally not confer expression throughout the plant under all conditions. Accordingly, a regulatable promoter sequence may be a promoter sequence that confers expression on a gene to which it is operably connected in a particular location within the plant or alternatively, throughout the plant under a specific set of conditions, 35 such as following induction of gene expression by a chemical compound or other elicitor.

Preferably, the regulatable promoter used in the performance of the present invention confers expression in a specific location within the plant, either constitutively or following

induction, however not in the whole plant under any circumstances. Included within the scope of such promoters are cell-specific promoter sequences, tissue-specific promoter sequences, organ-specific promoter sequences, cell cycle specific gene promoter sequences, inducible promoter sequences and constitutive promoter sequences that have  
5 been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within a transposable genetic element (*Ac*, *Ds*, *Spm*, *En*, or other transposon). Those skilled in the art will be aware that an "inducible promoter" is a promoter the transcriptional activity of which is increased or induced in response to a developmental, chemical, environmental, or physical stimulus. Similarly, the  
10 skilled craftsman will understand that a "constitutive promoter" is a promoter that is transcriptionally active throughout most, but not necessarily all parts of an organism, preferably a plant, during most, but not necessarily all phases of its growth and development.

Generally by "weak promoter" is intended a promoter that drives expression of a coding  
15 sequence at a low level. By "low level" is intended at levels of about 1/10,000 transcripts to about 1/100,000 transcripts, to about 1/500,000 transcripts. Conversely, a "strong promoter" drives expression of a coding sequence at high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

The term "cell-specific" shall be taken to indicate that expression is predominantly in a  
20 particular cell or cell-type, preferably of plant origin, albeit not necessarily exclusively in said cell or cell-type.

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular tissue or tissue-type, preferably of plant origin, albeit not necessarily exclusively in said tissue or tissue-type.

25 Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular organ, preferably of plant origin, albeit not necessarily exclusively in said organ. "Root-specific" means that the promoter is expressed in the root only and not in other tissues of the plant.

By "root-preferred" it is intended that expression of the heterologous nucleotide sequence  
30 is most abundant in root, but could also have low expression levels elsewhere in the plant. While some level of expression of the heterologous nucleotide sequence occurs in other plant tissue types, expression occurs most abundantly in the root including primary, lateral and adventitious roots.

By "root" is intended any part of the root structure, comprising the root cap, apical  
35 meristem, protoderm, ground meristem, procambium, endodermis, cortex, vascular cortex, epidermis, and the like.

The term "cell cycle specific" shall be taken to indicate that expression is predominantly cyclic and occurring in one or more, not necessarily consecutive phases of the cell cycle albeit not necessarily exclusively in cycling cells, preferably of plant origin.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence, or in  
5 operable connection with a promoter sequence means positioning said nucleic acid molecule such that expression is controlled by the promoter sequence. A promoter is usually, but not necessarily, positioned upstream, or at the 5'-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates. In the construction of heterologous promoter/structural gene combinations it is generally  
10 preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect  
15 to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

"Expression" means the production of a protein or nucleotide sequence in the cell itself or in a cell-free system. It includes transcription into an RNA product, post-transcriptional  
20 modification and/or translation to a protein product or polypeptide from a DNA encoding that product, as well as possible post-translational modifications.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence  
25 "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. In case the control sequence is a promoter, it is obvious for a skilled person that double-stranded nucleic acid is preferably used.

Examples of promoters suitable for use in gene constructs of the present invention include those listed in Table 4, amongst others. The promoters listed in Table 4 are provided for  
30 the purposes of exemplification only and the present invention is not to be limited by the list provided therein.

In the case of constitutive promoters or promoters that induce expression throughout the entire plant, it is preferred that such sequences are modified by the addition of nucleotide sequences derived from one or more of the tissue-specific promoters listed in Table 4, or  
35 alternatively, nucleotide sequences derived from one or more of the above-mentioned tissue-specific inducible promoters, to confer tissue-specificity thereon. For example, the *CaMV 35S* promoter may be modified by the addition of maize *Adh1* promoter sequence,



to confer anaerobically-regulated root-specific expression thereon, as described previously (Ellis *et al.* 1987). Another example describes conferring root specific or root abundant gene expression by fusing the *CaMV35S* promoter to elements of the maize glycine-rich protein GRP3 gene (Feix and Wulff 2000 - WO0015662). Such modifications  
5 can be achieved by routine experimentation by those skilled in the art.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signal termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from  
10 viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the gene constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene  
15 terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene terminator sequence, the Cauliflower mosaic virus (*CaMV*) 35S gene terminator sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays* zein gene terminator sequence, the *rbcS-1A* gene terminator, and the *rbcS-3A* gene terminator sequences, amongst others.

20 Those skilled in the art will readily be in a position to provide additional promoters and terminators that are useful in performing the present invention.

**Table 4. Exemplary plant-expressible promoters for use  
in the performance of the present invention**

<b>I: CELL-SPECIFIC, TISSUE-SPECIFIC, AND ORGAN-SPECIFIC PROMOTERS</b>		
<b>GENE SOURCE</b>	<b>EXPRESSION PATTERN</b>	<b>REFERENCE</b>
$\alpha$ -amylase ( <i>Amy32b</i> )	aleurone	Lanahan <i>et al</i> , Plant Cell 4:203-211, 1992; Skriver <i>et al</i> , Proc Natl Acad Sci USA 88:7266-7270, 1991
cathepsin $\beta$ -like gene	aleurone	Cejudo <i>et al</i> , Plant Mol Biol 20:849-856, 1992
<i>Agrobacterium</i> <i>rhizogenes rolB</i>	cambium	Nilsson <i>et al</i> , Physiol Plant 100:456-462, 1997
AtPRP4	flowers	<a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a>
chalcone synthase (chsA)	flowers	Van der Meer <i>et al</i> , Plant Mol Biol 15:95- 109, 1990
LAT52	anther	Twell <i>et al</i> , Mol Gen Genet 217:240-245, 1989
<i>apetala-3</i>	flowers	
chitinase	fruit (berries, grapes, etc)	Thomas <i>et al</i> . CSIRO Plant Industry, Urrbrae, South Australia, Australia; <a href="http://winetitles.com.au/qwrdc/csh95-1.html">http://winetitles.com.au/qwrdc/csh95-1.html</a>
rbcs-3A	green tissue (eg leaf)	Lam <i>et al</i> , Plant Cell 2:857-866, 1990; Tucker <i>et al</i> , Plant Physiol 113:1303-1308, 1992
leaf-specific genes	leaf	Baszczynski <i>et al</i> , Nucl Acid Res 16:4732, 1988
AtPRP4	leaf	<a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a>
chlorella virus adenine methyltransferase gene promoter	leaf	Mitra and Higgins, Plant Mol Biol 26:85-93, 1994
aldP gene promoter from rice	leaf	Kagaya <i>et al</i> , Mol Gen Genet 248:668-674, 1995
rbcs promoter from rice or tomato	leaf	Kyozuka <i>et al</i> , Plant Physiol 102:991-1000, 1993
<i>Pinus cab-6</i>	leaf	Yamamoto <i>et al</i> , Plant Cell Physiol 35:773-

		778, 1994
rubisco promoter	leaf	
cab (chlorophyll a/b/binding protein	leaf	
pea Blec4 gene	vegetative and floral epidermal tissues	Mandaci and Dobres, Plant Mol Biol 34:961-965
SAM22	senescent leaf	Crowell <i>et al</i> , Plant Mol Biol 18:459-466, 1992
<i>ltp gene (lipid transfer gene)</i>		Fleming <i>et al</i> , Plant J 2:855-862, 1992
<i>R. japonicum nif</i> gene	nodule	United States Patent No 4 803165
<i>B. japonicum nifH</i> gene	nodule	United States Patent No 5008194
GmENOD40	nodule	Yang <i>et al</i> , Plant J 3:573-585, 1993
PEP carboxylase (PEPC)	nodule	Pathirana <i>et al</i> , Plant Mol Biol 20:437-450, 1992
leghaemoglobin (Lb)	nodule	Gordon <i>et al</i> , J Exp Bot 44:1453-1465, 1993
<i>Tungro bacilliform</i> virus gene	phloem	Bhattacharyya-Pakrasi <i>et al</i> , Plant J 4:71-79, 1992
pollen-specific genes	pollen; microspore	Albani <i>et al</i> , Plant Mol Biol 15:605, 1990; Albani <i>et al</i> , Plant Mol Biol 16:501, 1991
Zm13	pollen	Guerrero <i>et al</i> , Mol Gen Genet 224:161-168, 1993
apg gene	microspore	Twell <i>et al</i> , Sex Plant Reprod 6:217-224, 1993
maize pollen-specific gene	pollen	Hamilton <i>et al</i> , Plant Mol Biol 18:211-218, 1992
sunflower pollen-expressed gene	pollen	Baltz <i>et al</i> , Plant J 2:713-721, 1992
<i>B. napus</i> pollen-specific gene	pollen; anther; tapetum	Arnoldo <i>et al</i> , J Cell Biochem, Abstract No. Y101, 204, 1992
root-expressible genes	roots	Tingey <i>et al</i> , EMBO J 6:1, 1987
tobacco auxin-inducible gene	root tip	Van der Zaal <i>et al</i> , Plant Mol Biol 16:983, 1991
$\beta$ -tubulin	root	Oppenheimer <i>et al</i> , Gene 63:87, 1988
tobacco root-specific	root	Conkling <i>et al</i> , Plant Physiol 93:1203, 1990

genes		
<i>B. napus</i> G1-3b gene	root	United States Patent No 5401836
SbPRP1	roots	Suzuki <i>et al</i> , Plant Mol Biol 21:109-119, 1993
AtPRP1; AtPRP3	roots; root hairs	<a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a>
RD2 gene	root cortex	<a href="http://www2.cnsu.edu/ncsu/research">http://www2.cnsu.edu/ncsu/research</a>
TobRB7 gene	root vasculature	<a href="http://www2.cnsu.edu/ncsu/research">http://www2.cnsu.edu/ncsu/research</a>
AtPRP4	leaves; flowers; lateral root primordia	<a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a>
seed-specific genes	seed	Simon <i>et al</i> , Plant Mol Biol 5:191, 1985; Scofield <i>et al</i> , J Biol Chem 262:12202, 1987; Baszczynski <i>et al</i> , Plant Mol Biol 14:633, 1990
Brazil Nut albumin	seed	Pearson <i>et al</i> , Plant Mol Biol 18:235-245, 1992
legumin	seed	Ellis <i>et al</i> , Plant Mol Biol 10:203-214, 1988
glutelin (rice)	seed	Takaiwa <i>et al</i> , Mol Gen Genet 208:15-22, 1986; Takaiwa <i>et al</i> , FEBS Lett 221:43-47, 1987
zein	seed	Matzke <i>et al</i> , Plant Mol Biol 14:323-32 1990
napA	seed	Stalberg <i>et al</i> , Planta 199:515-519, 1996
wheat LMW and HMW glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; Nucl Acids Res 17:461-462, 1989
wheat SPA	seed	Albani <i>et al</i> , Plant Cell 9:171-184, 1997
cZ19B1, maize 19 kDa zein	seed	WO0011177
mi1ps, maize myoinositol-1-Pi synthase	seed	WO0011177
wheat $\alpha$ , $\beta$ , $\gamma$ -gliadins	endosperm	EMBO J 3:1409-1415, 1984
barley <i>ltr1</i> promoter	endosperm	
barley B1, C, D, hordein	endosperm	Theor Appl Gen 98:1253-1262, 1999; Plant J 4:343-355, 1993; Mol Gen Genet 250:750-60, 1996
barley DOF	endosperm	Mena <i>et al</i> , Plant J 116:53-62, 1998
<i>blz2</i>	endosperm	EP99106056.7
synthetic promoter	endosperm	Vicente-Carbajosa <i>et al</i> , Plant J 13:629-640, 1998

rice prolamin NRP33	endosperm	Wu <i>et al</i> , Plant Cell Physiol 39: 885-889, 1998
rice $\alpha$ -globulin G1b-1	endosperm	Wu <i>et al</i> , Plant Cell Physiol 39:885-889, 1998
maize END genes	endosperm	WO0012733
barley END1	endosperm	WO9808961
barley NUC1	nucellus	WO9808961
rice OSH1	embryo	Sato <i>et al</i> , Proc Natl Acad Sci USA 93:8117-8122, 1996
rice $\alpha$ -globulin REB/OHP-1	endosperm	Nakase <i>et al</i> , Plant Mol Biol 33:513-522, 1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-168, 1997
maize ESR gene family	endosperm	Plant J 12:235-246, 1997
sorgum $\gamma$ -kafirin	endosperm	Plant Mol Biol 32:1029-1035, 1996
KNOX	embryo	Postma-Haarsma <i>et al</i> , Plant Mol Biol 39:257-271, 1999
rice oleosin	embryo and aleuron	Wu <i>et al</i> , J Biochem 123:386, 1998
sunflower oleosin	seed (embryo and dry seed)	Cummins <i>et al</i> , Plant Mol Biol 19:873-876, 1992
LEAFY	shoot meristem	Weigel <i>et al</i> , Cell 69:843-859, 1992
<i>Arabidopsis thaliana</i> knat1	shoot meristem	Accession number AJ131822
	shoot meristem	Accession number Z71981
CLAVATA1	shoot meristem	Accession number AF049870
stigma-specific genes	stigma	Nasrallah <i>et al</i> , Proc Natl Acad Sci USA 85:5551, 1988; Trick <i>et al</i> , Plant Mol Biol 15:203, 1990
class I patatin gene	tuber	Liu <i>et al</i> , Plant Mol Biol 153:386-395, 1991
PCNA rice	meristem	Kosugi <i>et al</i> , Nucl Acids Res 19:1571-1576, 1991; Kosugi and Ohashi, Plant Cell 9:1607-1619, 1997

Pea TubA1 tubulin	Dividing cells	Stotz and Long, Plant Mol Biol 41:601-614, 1999
<i>Arabidopsis</i> cdc2a	cycling cells	Chung and Parish, FEBS Lett 362:215-219, 1995
<i>Arabidopsis</i> Rop1A	Anthers; mature pollen + pollen tubes	Li <i>et al</i> , Plant Physiol 118:407-417, 1998

<i>Arabidopsis</i> AtDMC1	Meiosis-associated	Klimyuk and Jones, Plant J 11:1-14, 1997
Pea PS-IAA4/5 and PS-IAA6	Auxin-inducible	Wong <i>et al</i> , Plant J 9:587-599, 1996
Pea farnesyltransferase	Meristematic tissues; phloem near growing tissues; light- and sugar-repressed	Zhou <i>et al</i> , Plant J 12:921-930, 1997
Tobacco ( <i>N. sylvestris</i> ) cyclin B1;1	Dividing cells / meristematic tissue	Trehin <i>et al</i> , Plant Mol.Biol. 35:667-672, 1997
<i>Catharanthus roseus</i> Mitotic cyclins CYS (A-type) and CYM (B-type)	Dividing cells / meristematic tissue	Ito <i>et al</i> , Plant J 11:983-992, 1997
<i>Arabidopsis</i> cyc1At (=cyc B1;1) and cyc3aAt (A-type)	Dividing cells / meristematic tissue	Shaul <i>et al</i> , Proc Natl Acad Sci USA 93:4868-4872, 1996
<i>Arabidopsis</i> tef1 promoter box	Dividing cells / meristematic tissue	Regad <i>et al</i> , Mol Gen Genet 248:703-711, 1995
<i>Catharanthus roseus</i> cyc07	Dividing cells / meristematic tissue	Ito <i>et al</i> , Plant Mol Biol 24:863-878, 1994

## II: EXEMPLARY CONSTITUTIVE PROMOTERS

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
Actin	constitutive	McElroy <i>et al</i> , Plant Cell 2:163-171, 1990
CAMV 35S	constitutive	Odell <i>et al</i> , Nature 313:810-812, 1985
CaMV 19S	constitutive	Nilsson <i>et al</i> , Physiol Plant 100:456-462, 1997
GOS2	constitutive	de Pater <i>et al</i> , Plant J 2:837-844, 1992
ubiquitin	constitutive	Christensen <i>et al</i> , Plant Mol Biol 18:675-689, 1992
rice cyclophilin	constitutive	Buchholz <i>et al</i> , Plant Mol Biol 25:837-843, 1994
maize histone H3	constitutive	Lepetit <i>et al</i> , Mol Gen Genet 231:276-285, 1992
alfalfa histone H3	constitutive	Wu <i>et al</i> , Nucleic Acids Res 17:3057-3063, 1989; Wu <i>et al</i> , Plant Mol Biol 11:641-649, 1988

actin 2	constitutive	An <i>et al</i> , Plant J 10:107-121, 1996
<b>III: EXEMPLARY STRESS-INDUCIBLE PROMOTERS</b>		
<b>NAME</b>	<b>STRESS</b>	<b>REFERENCE</b>
P5CS (delta(1)-pyrroline-5-carboxylate syntase)	salt, water	Zhang <i>et al</i> , Plant Sci 129:81-89, 1997
cor15a	cold	Hajela <i>et al</i> , Plant Physiol 93:1246-1252, 1990
cor15b	cold	Wiihelm <i>et al</i> , Plant Mol Biol 23:1073-1077, 1993
cor15a (-305 to +78 nt)	cold, drought	Baker <i>et al</i> , Plant Mol Biol 24: 01-713, 1994
rd29	salt, drought, cold	Kasuga <i>et al</i> , Nature Biotechnol 18:287-291, 1999
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros <i>et al</i> , Plant Mol Biol 19 665-75, 1992. Marrs <i>et al</i> , Dev Genet 14:27-41, 1993. Schoffl <i>et al</i> , Mol Gen Genet 217:246-53, 1989.
smHSP (small heat shock proteins)	heat	Waters <i>et al</i> , J Exp Bot 47:325-338, 1996
wcs120	cold	Ouellete <i>et al</i> , FEBS Lett 423:324-328, 1998
ci7	cold	Kirch <i>et al</i> , Plant Mol Biol 33:897-909, 1997
Adh	cold, drought, hypoxia	Dolferus <i>et al</i> , Plant Physiol 105:1075-87, 1994
pws18	salt and drought	Joshee <i>et al</i> , Plant Cell Physiol 39:64-72, 1998
ci21A	cold	Schneider <i>et al</i> , Plant Physiol 113:335-45, 1997
Trg-31	drought	Chaudhary <i>et al</i> , Plant Mol Biol 30:1247-57, 1996
osmotin	osmotic	Raghothama <i>et al</i> , Plant Mol Biol 23:1117-28, 1993
lapA	wounding, enviromental	WO99/03977 University of California/INRA

IV: EXEMPLARY PATHOGEN-INDUCIBLE PROMOTERS		
NAME	PATHOGEN	REFERENCE
RB7	Root-knot nematodes ( <i>Meloidogyne</i> spp.)	US5760386 - North Carolina State University; Opperman <i>et al</i> , Science 263:221-23, 1994
PR-1, 2, 3, 4, 5, 8, 11	fungus, viral, bacterial	Ward <i>et al</i> , Plant Cell 3:1085-1094, 1991; Reiss <i>et al</i> 1996; Lebel <i>et al</i> , Plant J 16:223-233, 1998; Melchers <i>et al</i> , Plant J 5:469-480, 1994; Lawton <i>et al</i> , Plant Mol Biol, 19:735-743, 1992
HMG2	nematodes	WO9503690 - Virginia Tech Intellectual Properties Inc .
Abi3	Cyst nematodes ( <i>Heterodera</i> spp.)	unpublished
ARM1	nematodes	Barthels <i>et al</i> , Plant Cell 9:2119-2134, 1997 WO 98/31822 - Plant Genetic Systems
Att0728	nematodes	Barthels <i>et al</i> , Plant Cell 9: 2119-2134, 1997 PCT/EP98/07761
Att1712	nematodes	Barthels <i>et al</i> , Plant Cell 9, 2119-2134, 1997 PCT/EP98/07761
Gst1	Different types of pathogens	Strittmatter <i>et al</i> , Mol Plant-Microbe Interact 9:68-73, 1996
LEMMI	nematodes	WO 92/21757 - Plant Genetic Systems
CLE	geminivirus	PCT/EP99/03445 - CINESTAV
PDF1.2	Fungal including <i>Alternaria brassicicola</i> and <i>Botrytis cinerea</i>	Manners <i>et al</i> , Plant Mol Biol, 38:1071-1080, 1998
Thi2.1	Fungal - <i>Fusarium oxysporum</i> f sp. <i>matthiolae</i>	Vignutelli <i>et al</i> , Plant J 14:285-295, 1998
DB#226	nematodes	Bird and Wilson, Mol Plant-Microbe Interact 7:419-442, 1994 WO 95.322888
DB#280	nematodes	Bird and Wilson, Mol Plant-Microbe Interact 7:419-442, 1994 WO 95.322888
Cat2	Nematodes	Niebel <i>et al</i> , Mol Plant-Microbe Interact 8:371-



		378, 1995
QTub	Nematodes	Aristizabal <i>et al</i> (1996), 8 <sup>th</sup> International Congress on Plant-Microbe Interaction, Knoxville US B-29
sHSP	Nematodes	Fenoll <i>et al</i> (1997) In: Cellular and molecular aspects of plant-nematode interactions. Kluwer Academic, C. Fenoll, F.M.W. Grundler and S.A. Ohi (Eds.),
Tsw12	Nematodes	Fenoll <i>et al</i> (1997) In: Cellular and molecular aspects of plant-nematode interactions. Kluwer Academic, C. Fenoll, F.M.W. Grundler and S.A. Ohi (Eds.)
Hs1(pro1)	Nematodes	WO 98/122335 – Jung
nsLTP	viral, fungal, bacterial	Molina and Garcia-Olmedo FEBS Lett, 316:119-122, 1993
RIP	viral, fungal	Tumer <i>et al</i> , Proc Natl Acad Sci USA 94:3866-3871, 1997

In the context of the current invention, "ectopic expression" or "ectopic overexpression" of a gene or a protein are conferring to expression patterns and/or expression levels of said gene or protein normally not occurring under natural conditions. Ectopic expression can be achieved in a number of ways including operably linking of a coding sequence encoding said protein to an isolated homologous or heterologous promoter in order to create a chimeric gene and/or operably linking said coding sequence to its own isolated promoter (i.e. the unisolated promoter naturally driving expression of said protein) in order to create a recombinant gene duplication or gene multiplication effect.

"Ectopic expression" not only can result in overexpression of a gene but can also result in "downregulation of expression", for instance of the homologous gene in the plant where expression is effected.

With "ectopic co-expression" is meant the ectopic expression or ectopic overexpression of two or more genes or proteins. The same or, more preferably, different promoters are used to confer expression of said genes or proteins.

Preferably, the promoter sequence used in the context of the present invention is operably linked to a coding sequence or open reading frame (ORF) encoding one of the inventive sugar beet polypeptides or a homologue, derivative and/or an immunologically active fragment thereof as defined *supra*.

"Downregulation of expression" as used herein means lowering levels of gene expression and/or levels of active gene product and/or levels of gene product activity. Decreases in expression may be accomplished by e.g. the addition of coding sequences or parts thereof in a sense orientation (if resulting in co-suppression) or in an antisense orientation

relative to a promoter sequence and furthermore by e.g. insertion mutagenesis (e.g. T-DNA insertion or transposon insertion) or by gene silencing strategies as described by e.g. Angell and Baulcombe 1998 (WO9836083), Lowe *et al.* 1989 (WO9853083), Lederer *et al.* 1999 (WO9915682) or Wang *et al.* 1999 (WO9953050). Genetic constructs aimed at  
5 silencing gene expression may have the nucleotide sequence of said gene (or one or more parts thereof) contained therein in a sense and/or antisense orientation relative to the promoter sequence. Another method to downregulate gene expression comprises the use of ribozymes, e.g. as described in Atkins *et al.* 1994 (WO9400012), Lenée *et al.* 1995 (WO9503404), Lutziger *et al.* 2000 (WO0000619), Prinsen *et al.* 1997 (WO9713865) and  
10 Scott *et al.* 1997 (WO9738116).

Modulating, including lowering, the level of active gene products or of gene product activity can be achieved by administering or exposing cells, tissues, organs or organisms to said gene product, a homologue, analogue, derivative and/or immunologically active fragment thereof. Immunomodulation is another example of a technique capable of  
15 downregulation levels of active gene product and/or of gene product activity and comprises administration of or exposing to or expressing antibodies to said gene product to or in cells, tissues, organs or organisms wherein levels of said gene product and/or gene product activity are to be modulated. Such antibodies comprise "plantibodies", single chain antibodies, IgG antibodies and heavy chain camel antibodies as well as fragments  
20 thereof.

Modulating, including lowering, the level of active gene products or of gene product activity can furthermore be achieved by administering or exposing cells, tissues, organs or organisms to an inhibitor or activator of said gene product or the activity thereof. Such inhibitors or activators include proteins (comprising e.g. proteinases and kinases) and  
25 chemical compounds identified according to the current invention as described *supra*.

In the context of the invention the term "agonist" refers to a substance that can be either a protagonist or an antagonist, i.e. can have either positive or negative effects, can be an enhancer or an inhibitor or a modulator as well.

In the context of the current invention is envisaged the downregulation of the expression  
30 the inventive sugar beet genes as defined higher. The invention further comprises downregulation of levels of the activity of the inventive sugar beet polypeptides whereby the inventive sugar beet polypeptides have been defined *supra*.

By "cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology" is meant that one or more developmental and/or morphological and/or  
35 biochemical and/or physiological characteristics of a plant is altered by the performance of one or more steps pertaining to the invention described herein.

"Cell fate" refers to the cell-type or cellular characteristics of a particular cell that are produced during plant development or a cellular process therefore, in particular during the cell cycle or as a consequence of a cell cycle process.

"Plant development" or the term "plant developmental characteristic" or similar term shall, when used herein, be taken to mean any cellular process of a plant that is involved in determining the developmental fate of a plant cell, in particular the specific tissue or organ type into which a progenitor cell will develop. Cellular processes relevant to plant development will be known to those skilled in the art. Such processes include, for example, morphogenesis, photomorphogenesis, shoot development, root development, vegetative development, reproductive development, stem elongation, flowering, and regulatory mechanisms involved in determining cell fate, in particular a process or regulatory process involving the cell cycle.

"Plant morphology" or the term "plant morphological characteristic" or similar term will, when used herein, be understood by those skilled in the art to refer to the external appearance of a plant, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, number, position, colour, texture, arrangement, and patternation of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, stem, leaf, shoot, petiole, trichome, flower, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, fruit, cambium, wood, heartwood, parenchyma, aerenchyma, sieve element, phloem or vascular tissue, amongst others.

"Plant biochemistry" or the term "plant biochemical characteristic" or similar term will, when used herein, be understood by those skilled in the art to refer to the metabolic and catalytic processes of a plant, including primary and secondary metabolism and the products thereof, including any small molecules, macromolecules or chemical compounds, such as but not limited to starches, sugars, proteins, peptides, enzymes, hormones, growth factors, nucleic acid molecules, celluloses, hemicelluloses, calloses, lectins, fibres, pigments such as anthocyanins, vitamins, minerals, micronutrients, or macronutrients, that are produced by plants.

"Plant physiology" or the term "plant physiological characteristic" or similar term will, when used herein, be understood to refer to the functional processes of a plant, including developmental processes such as growth, expansion and differentiation, sexual development, sexual reproduction, seed set, seed development, grain filling, asexual reproduction, cell division, dormancy, germination, light adaptation, photosynthesis, leaf expansion, fiber production, secondary growth or wood production, amongst others; responses of a plant to externally-applied factors such as metals, chemicals, hormones, growth factors, environment and environmental stress factors (eg. anoxia, hypoxia, high

temperature, low temperature, dehydration, light, daylength, flooding, salt, heavy metals, amongst others), including adaptive responses of plants to said externally-applied factors.

“Stress” or “environmental stress” is a circumstance caused by elements present in the environment which may include but are not limited to drought, salt, dehydration, heat, cold, freezing, water logging, wounding, mechanical stress, oxidative stress, ozone, high light heavy metals, nutrient deprivation, toxic chemicals, pathogen (including viruses, bacteria, fungi, insects and nematodes) and combinations of these.

“Osmotic stress” is any kind of stress which alters the osmotic potential in the cell. For example osmotic stress can be caused by drought or salt or frost.

“Non-environmental stress” is a circumstance caused by elements or factors from the organism (e.g. gene defect).

As used herein, “stress tolerance” refers to the capacity to grow and produce biomass during stress, the capacity to reinitiate growth and biomass production after stress, and the capacity to survive stress. The term “stress tolerance” also covers the capacity of the plant to undergo its developmental program during stress similarly to under non-stressed conditions, e.g. to switch from dormancy to germination and from vegetative to reproductive phase under stressed conditions similarly as under non-stressed conditions.

Furthermore it is shown that genes protecting against osmotic stress (like trehalose) also protect against oxidative stress (Benaroudj *et al.* 2001). Therefore a person skilled in the art can assume that when an isolated gene confers salt tolerance to a host organism when transfected herein, it could also confer oxidative stress tolerance. Oxidative stress occurs in situations of cold stress combined with high light or in situations of ozone stress, in case of necrosis as a result of pathogen infection or wounding, in case of senescence or by application of certain herbicides (like atrazine or paraquat). Since the function of many osmoprotectants is actually unknown and that mannitol for example also has been shown to function as a scavenger of oxygen radicals, it can be assumed that oxidative stress also occurs in case of osmotic stress.

Means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using  $\text{CaCl}_2$  and variations thereof, in particular the method described previously (Hanahan 1983), direct DNA uptake into protoplasts (Krens *et al.* 1982; Paszkowski *et al.* 1984), PEG-mediated uptake to protoplasts (Armstrong *et al.* 1990) microparticle bombardment, electroporation (Fromm *et al.* 1985), microinjection of DNA (Crossway *et al.* 1986; Fromm *et al.* 1985), microparticle bombardment of tissue explants or cells (Christou *et al.* 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially (An *et al.* 1985; Dodds 1985; Herrera-Estrella *et al.* 1983a; Herrera-Estrella *et al.* 1983b). Methods for transformation of monocotyledonous plants are well

known in the art and include *Agrobacterium*-mediated transformation (Cheng *et al.* 1997 - WO9748814; Hansen 1998 - WO9854961, Hiei *et al.* 1994 - WO9400977; Hiei *et al.* 1998 - WO9817813; Rikiishi *et al.* 1999 - WO9904618; Saito *et al.* 1995 - WO9506722), microprojectile bombardment (Adams *et al.* 1999 - US5969213; Bowen *et al.* 1998 - US5736369; Chang *et al.* 1994 - WO9413822; Lundquist *et al.* 1999 - US5874265/US5990390; Vasil and Vasil 1995 -US5405765; Walker *et al.* 1999 - US5955362), DNA uptake (Eyal *et al.* 1993 - WO9318168), microinjection of *Agrobacterium* cells (von Holt 1994 - DE4309203) and sonication (Finer *et al.* 1997 - US5693512).

10 For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5122466) and Sanford and Wolf (U.S. Patent No. 4945050). When using ballistic transformation procedures, the gene construct  
15 may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5  $\mu\text{m}$  gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in accordance  
20 with procedures well known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets  
25 include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots  
30 are developed sequentially from meristematic centers.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

Preferably, the plant is produced according to the inventive method is transfected or  
35 transformed with a genetic sequence, or amenable to the introduction of a protein, by any art-recognized means, such as microprojectile bombardment, microinjection, *Agrobacterium*-mediated transformation (including the 'flower dip' transformation method;

(Bechtold & Pelletier 1998; Trieu *et al.* 2000)), protoplast fusion, or electroporation, amongst others. Most preferably said plant is produced by *Agrobacterium*-mediated transformation.

The "seedling" is the juvenile plant that arises from the mature embryo after seed germination.

With "differentiation of a cell" it is understood that the cell develops unique features to be engaged for a specific function. Mostly differentiation is irreversible.

*Agrobacterium*-mediated transformation or agrolistic transformation of plants, yeast, moulds or filamentous fungi is based on the transfer of part of the transformation vector sequences, called the T-DNA, to the nucleus and on integration of said T-DNA in the genome of said eukaryote.

With "*Agrobacterium*" is meant a member of the *Agrobacteriaceae*, more preferably *Agrobacterium* or *Rhizobacterium* and most preferably *Agrobacterium tumefaciens*.

With "T-DNA", or transferred DNA, is meant that part of the transformation vector flanked by T-DNA borders which is, after activation of the *Agrobacterium vir* genes, nicked at the T-DNA borders and is transferred as a single stranded DNA to the nucleus of an eukaryotic cell.

When used herein, with "T-DNA borders", "T-DNA border region", or "border region" are meant either right T-DNA border (RB) or left T-DNA border (LB). Such a border comprises a core sequence flanked by a border inner region as part of the T-DNA flanking the border and/or a border outer region as part of the vector backbone flanking the border. The core sequences comprise 22 bp in case of octopine-type vectors and 25 bp in case of nopaline-type vectors. The core sequences in the right border region and left border region form imperfect repeats. Border core sequences are indispensable for recognition and processing by the *Agrobacterium* nicking complex consisting of at least VirD1 and VirD2. Core sequences flanking a T-DNA are sufficient to promote transfer of said T-DNA. However, efficiency of transformation using transformation vectors carrying said T-DNA solely flanked by said core sequences is low. Border inner and outer regions are known to modulate efficiency of T-DNA transfer (Wang *et al.* 1987). One element enhancing T-DNA transfer has been characterised and resides in the right border outer region and is called *overdrive* (Peralta *et al.* 1986; van Haaren *et al.* 1987).

With "T-DNA transformation vector" or "T-DNA vector" is meant any vector encompassing a T-DNA sequence flanked by a right and left T-DNA border consisting of at least the right and left border core sequences, respectively, and used for transformation of any eukaryotic cell.

With "T-DNA vector backbone sequence" or "T-DNA vector backbone sequences" is meant all DNA of a T-DNA containing vector that lies outside of the T-DNA borders and, more specifically, outside the nicking sites of the border core imperfect repeats.

The current invention includes optimised T-DNA vectors such that vector backbone integration in the genome of a eukaryotic cell is minimised or absent. With "optimised T-DNA vector" is meant a T-DNA vector designed either to decrease or abolish transfer of vector backbone sequences to the genome of a eukaryotic cell. Such T-DNA vectors are known to the one familiar with the art and include those described previously (Hanson *et al.* 1999, Stuiver *et al.* 1999 - WO9901563).

The current invention clearly considers the inclusion of a DNA sequence of the present invention encoding a DRE-binding factor DBF1, homologue, derivative or immunologically active fragment thereof as defined *supra*, in any T-DNA vector comprising binary transformation vectors, super-binary transformation vectors, co-integrate transformation vectors, Ri-derived transformation vectors as well as in T-DNA carrying vectors used in agrolistic transformation.

With "binary transformation vector" is meant a T-DNA transformation vector comprising: a T-DNA region comprising at least one gene of interest and/or at least one selectable marker active in the eukaryotic cell to be transformed; and a vector backbone region comprising at least origins of replication active in *Escherichia coli* and *Agrobacterium* and markers for selection in *Escherichia coli* and *Agrobacterium*. Alternatively, replication of the binary transformation vector in *Agrobacterium* is dependent on the presence of a separate helper plasmid. The binary vector pGreen and the helper plasmid pSoup form an example of such a system as described in e.g. (Hellens *et al.* 2000) or as available on the internet site <http://www.pgreen.ac.uk>.

The T-DNA borders of a binary transformation vector can be derived from octopine-type or nopaline-type Ti plasmids or from both. The T-DNA of a binary vector is only transferred to a eukaryotic cell in conjunction with a helper plasmid. Also known in the art are multiple binary vector *Agrobacterium* strains for efficient co-transformation of plants (Bidney and Scelonge 2000 - WO0018939).

With "helper plasmid" is meant a plasmid that is stably maintained in *Agrobacterium* and is at least carrying the set of *vir* genes necessary for enabling transfer of the T-DNA. Said set of *vir* genes can be derived from either octopine-type or nopaline-type Ti plasmids or from both.

With "super-binary transformation vector" is meant a binary transformation vector additionally carrying in the vector backbone region a *vir* region of the Ti plasmid pTiBo542 of the super-virulent *Agrobacterium tumefaciens* strain A281 (Hiei *et al.* 1994 -

EP0604662, Hiei *et al.* 1995 - EP0687730). Super-binary transformation vectors are used in conjunction with a helper plasmid.

With "co-integrate transformation vector" is meant a T-DNA vector at least comprising:  
a T-DNA region comprising at least one gene of interest and/or at least one selectable  
5 marker active in plants; and

a vector backbone region comprising at least origins of replication active in *Escherichia coli* and *Agrobacterium*, and markers for selection in *Escherichia coli* and *Agrobacterium*, and a set of *vir* genes necessary for enabling transfer of the T-DNA.

10 The T-DNA borders and said set of *vir* genes of a said T-DNA vector can be derived from either octopine-type or nopaline-type Ti plasmids or from both.

With "Ri-derived plant transformation vector" is meant a binary transformation vector in which the T-DNA borders are derived from a Ti plasmid and said binary transformation vector being used in conjunction with a 'helper' Ri-plasmid carrying the necessary set of *vir* genes.

15 As used herein, the term "selectable marker gene" or "selectable marker" or "marker for selection" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof. Suitable selectable marker genes contemplated herein include the ampicillin resistance (*Amp<sup>r</sup>*), tetracycline  
20 resistance gene (*Tc<sup>r</sup>*), bacterial kanamycin resistance gene (*Kan<sup>r</sup>*), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npII*), hygromycin resistance gene,  $\beta$ -glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al.* 1997), and luciferase gene, amongst others.

With "agrolistics", "agrolistic transformation" or "agrolistic transfer" is meant here a  
25 transformation method combining features of *Agrobacterium*-mediated transformation and of biolistic DNA delivery. As such, a T-DNA containing target plasmid is co-delivered with DNA/RNA enabling in planta production of VirD1 and VirD2 with or without VirE2 (Hansen & Chilton 1996; Hansen *et al.* 1997, Hansen and Chilton 1997 - WO9712046).

With "foreign DNA" is meant any DNA sequence that is introduced in the host's genome  
30 by recombinant techniques. Said foreign DNA includes e.g. a T-DNA sequence or a part thereof such as the T-DNA sequence comprising the selectable marker in an expressible format. Foreign DNA furthermore includes intervening DNA sequences as defined *supra*.

"Plant cell" comprises any cell derived from any plant and existing in culture as a single cell, a group of cells or a callus. A plant cell may also be any cell in a developing or  
35 mature plant in culture or growing in nature.

"Plant" or "Plants" comprise all plant species which belong to the superfamily *Viridiplantae*. The present invention is applicable to any plant, in particular monocotyledonous plants



and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia* spp., *Acer* spp., *Actinidia* spp., *Aesculus* spp., *Agathis australis*, *Albizia amara*, *Alsophila tricolor*, *Andropogon* spp., *Arachis* spp., *Areca catechu*, *Astelia fragrans*, *Astragalus cicer*, *Baikiaea plurijuga*, *Betula* spp., *Brassica* spp., *Bruguiera gymnorrhiza*, *Burkea africana*, *Butea frondosa*, *Cadaba farinosa*, *Calliandra* spp., *Camellia sinensis*, *Canna indica*, *Capsicum* spp., *Cassia* spp., *Centroema pubescens*, *Chaenomeles* spp., *Cinnamomum cassia*, *Coffea arabica*, *Colophospermum mopane*, *Coronilla varia*, *Cotoneaster serotina*, *Crataegus* spp., *Cucumis* spp., *Cupressus* spp., *Cyathea dealbata*, *Cydonia oblonga*, *Cryptomeria japonica*, *Cymbopogon* spp., *Cynthea dealbata*, *Cydonia oblonga*, *Dalbergia monetaria*, *Davallia divaricata*, *Desmodium* spp., *Dicksonia squarosa*, *Diheteropogon amplexans*, *Dioclea* spp., *Dolichos* spp., *Dorycnium rectum*, *Echinochloa pyramidalis*, *Ehrartia* spp., *Eleusine coracana*, *Eragrostis* spp., *Erythrina* spp., *Eucalyptus* spp., *Euclea schimperi*, *Eulalia villosa*, *Fagopyrum* spp., *Feijoa sellowiana*, *Fragaria* spp., *Flemingia* spp., *Freycinetia banksii*, *Geranium thunbergii*, *Ginkgo biloba*, *Glycine javanica*, *Gliricidia* spp., *Gossypium hirsutum*, *Grevillea* spp., *Guibourtia coleosperma*, *Hedysarum* spp., *Hemarthia altissima*, *Heteropogon contortus*, *Hordeum vulgare*, *Hyparrhenia rufa*, *Hypericum erectum*, *Hyperthelia dissoluta*, *Indigo incarnata*, *Iris* spp., *Leptarrhena pyrolifolia*, *Lespedeza* spp., *Lettuca* spp., *Leucaena leucocephala*, *Loudetia simplex*, *Lotonus bainesii*, *Lotus* spp., *Macrotyloma axillare*, *Malus* spp., *Manihot esculenta*, *Medicago sativa*, *Metasequoia glyptostroboides*, *Musa sapientum*, *Nicotianum* spp., *Onobrychis* spp., *Ornithopus* spp., *Oryza* spp., *Peltophorum africanum*, *Pennisetum* spp., *Persea gratissima*, *Petunia* spp., *Phaseolus* spp., *Phoenix canariensis*, *Phormium cookianum*, *Photinia* spp., *Picea glauca*, *Pinus* spp., *Pisum sativum*, *Podocarpus totara*, *Pogonarthria fleckii*, *Pogonarthria squarrosa*, *Populus* spp., *Prosopis cineraria*, *Pseudotsuga menziesii*, *Pterolobium stellatum*, *Pyrus communis*, *Quercus* spp., *Rhaphiolepis umbellata*, *Rhopalostylis sapida*, *Rhus natalensis*, *Ribes grossularia*, *Ribes* spp., *Robinia pseudoacacia*, *Rosa* spp., *Rubus* spp., *Salix* spp., *Schyzachyrium sanguineum*, *Sciadopitys verticillata*, *Sequoia sempervirens*, *Sequoiadendron giganteum*, *Sorghum bicolor*, *Spinacia* spp., *Sporobolus fimbriatus*, *Stiburus alopecuroides*, *Stylosanthos humilis*, *Tadehagi* spp., *Taxodium distichum*, *Themeda triandra*, *Trifolium* spp., *Triticum* spp., *Tsuga heterophylla*, *Vaccinium* spp., *Vicia* spp., *Vitis vinifera*, *Watsonia pyramidata*, *Zantedeschia aethiopica*, *Zea mays*, amaranth, artichoke, asparagus, broccoli, brussel sprout, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugarbeet, sugar cane, sunflower, tomato, squash, and tea, amongst others, or the seeds of any plant specifically named above or a tissue, cell or organ culture of any of the above species.

"Cereal" comprises crop plants with edible grain for example plants belonging to the grass family that is cultivated for its nutritious grains such as oats, barley, rye, wheat, rice, and corn etc.

With "yeast two-hybrid assay" is meant an assay that is based on the observation that many eukaryotic transcription factors comprise two domains, a DNA-binding domain (DB) and an activation domain (AD) which, when physically separated (i.e. disruption of the covalent linkage) do not effectuate target gene expression. Two proteins able to interact physically with one of said proteins fused to DB and the other of said proteins fused to AD will re-unite the DB and AD domains of the transcription factor resulting in target gene expression. The target gene in the yeast two-hybrid assay is usually a reporter gene such as the  $\beta$ -galactosidase gene. Interaction between protein partners in the yeast two-hybrid assay can thus be quantified by measuring the activity of the reporter gene product (Bartel & Fields 1997). Alternatively, a mammalian two-hybrid system can be used which includes e.g. a chimeric green fluorescent protein encoding reporter gene (Shioda *et al.* 2000). Yet another alternative consists of a bacterial two-hybrid system using e.g. *HIS* as reporter gene (Joung *et al.* 2000).

The term "fragment of a sequence" or "part of a sequence" means a truncated sequence of the original sequence referred to. The truncated sequence (nucleic acid or protein sequence) can vary widely in length; the minimum size being a sequence of sufficient size to provide a sequence with at least a comparable function and/or activity or the original sequence referred to, while the maximum size is not critical. In some applications, the maximum size usually is not substantially greater than that required to provide the desired activity and/or function(s) of the original sequence. Typically, the truncated amino acid will range from about 5 to about 333 amino acids or nuin length. More typically, however, the sequence will be a maximum of about 333 amino acids in length, preferably a maximum of about 330 amino acids. It is usually desirable to select sequences of at least about 10, 12 or 15 amino acids, or 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275 up to a maximum of about 300 or 325 amino acids. For instance, the truncated nucleic acid will correspond in length with the amino acid fragment it encodes.

Said compound or plurality of compounds may be comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compound(s) may be known in the art but hitherto not known to be capable of suppressing or activating cell cycle interacting proteins. The reaction mixture may be a cell free extract of may comprise a cell or tissue culture. Suitable set ups for the method of the invention are known to the person skilled in the art and are, for example, generally described previously (Alberts *et al.* 1994), in particular Chapter 17. The plurality of compounds may be, e.g., added to the reaction mixture, culture medium or injected into the cell.

- If a sample containing a compound or a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of acting as an agonist, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances or similar chemical and/or physical properties, and most preferably said substances are identical. Preferably, the compound identified according to the above-described method or its derivative is further formulated in a form suitable for the application in plant breeding or plant cell and tissue culture.
- 15 The present invention is further described by reference to the following non-limiting figures and examples.

## FIGURE LEGENDS

## Figure 1

Amino acid alignments of BvCKA2 with the catalytic subunit of other CK2. The accession number and sources of each of the CK2 are as follows: AtCKA2 (*Arabidopsis thaliana*,  
5 Acc. number Q08466), ZmCKA2 (*Zea mays*, Acc. number P28523), RnCKA1 (*Ratus norvegicus* Ac. number P19139), XICKA2 (*Xenopus laevis*, Ac. number P28020) and ScCKA2 (*Saccharomyces cerevisiae* Ac. number P19454). The identical amino acids are shaded. The boxed regions indicate important conserved residues in CKII. These include the motif 71-DWG-73 forming the catalytic site, the essential 63K and the highly basic 69-  
10 KKKKIKR-75 domain.

## Figure 2

Southern blot analysis of *BvCKA2* gene. Genomic DNA was digested with *Bam* HI (B), *Hind* III (H) and *Eco* RI (E) and separated on a 0.8 % agarose gel. The DNA was then transferred onto a nylon membrane and UV cross-linked. Hybridization was performed  
15 under high-stringency conditions (see example 8) using two radiolabeled probes: a fragment of 887 bp included in the coding region of *BvCKA2* cDNA (left panel), and a fragment of 323 bp corresponding to the 3 untranslated region of *BvCKA2* cDNA (right panel).

## Figure 3

20 *BvCKA2* complements the yeast double mutant *cka1 cka2*. The casein kinase thermosensitive yeast strain YDH8 (*cka1 cka2*) was transformed with either plasmid pYPGE15-*BvCKA2* (4,5,6,7) or the empty plasmid (1,2,3) and grown at the permissive temperature of 25° C (left) or at the thermosensitive temperature of 37° C (right)

## Figure 4

25 Increased tolerance to NaCl stress in yeast by overexpression of *ScCKA2* and *BvCKA2*. The yeast mutant strain JM26 was transformed with either the empty plasmid (pYPGE15) or the plasmid containing inserts encoding for *ScCKA2* or *BvCKA2*. Transformed cells were tested for salt tolerance as described in example 7. Plates contained SD medium with leucine, adenine and 150 mM NaCl when indicated.

30

## Figure 5

*BvCKA2* gene expression is up-regulated by NaCl stress. Northern analysis was performed as described in example 8. RNA was isolated from 3 week old sugar beet

leaves at 0, 3, 6, 8 and 24 hours after growing with (+) or without (-) 250 mM NaCl. The same RNA blot was hybridized with a 3' UTR fragment of *BvCKA2* and with an  $\alpha$  3 tubulin probe (*AtTUBA*, Ac. number M17189) from *Arabidopsis* used as control of filter transfer. Control of gel loading was done with ethidium bromide staining and is shown by the 3.5 Kb band of rRNA.

#### Figure 6

SEQ ID NO 1 to 5: DNA sequences of the nucleic acids of the invention, start and stop codons are underlined. SEQ ID NO 6 to 10: amino acid sequences of the polypeptides of the invention.

#### Figure 7

*In vitro* evaluation of the tolerance to NaCl of transgenic *Arabidopsis* expressing clone 154 cDNA (*BcCKA2*)

Culture medium: MS, NaCl concentrations: 0, 100 and 125 mM NaCl, Variables studied: % of plants with true leaves (A) and % of plants with cotyledons (B), Number of repetitions of the experiment : 6-8, Number of plants per concentration: 150-200, Statistical analysis: ANOVA, Confidence level: 99%

#### Figure 8

Picture of *Arabidopsis* seedlings grown on MS médium supplemented with 100mM NaCl. The seedlings were not transformed or transformed with the empty vector pBI121 or transformed with the 145 cDNA encoding *BvCKA2*.

#### Figure 9

Quantification of radioactive phenylalanin incorporation into proteins of plants transfected with *BvelF-1A*.

**EXAMPLES****Example 1: Plant material**

Sugar beet seeds (*Beta vulgaris* var. DITA) were sown on pots containing a mixture of sand and vermiculite (1:1 w/w). The plants were grown under greenhouse conditions (8 hours at 20 °C, 16 hours at 25°C with supplementary lighting to stimulate a minimum of 12 hours photoperiod). They were periodically irrigated with a nutrient solution containing 2.4 g/l  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1 g/l  $\text{KNO}_3$ , 1 g/l  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3 g/l  $\text{KH}_2\text{PO}_4$ , 5.6 mg/l Fe-quelate (Kelantren, Bayer), 1.1 mg/l  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 3.3 mg/l  $\text{MnO}_4 \cdot \text{H}_2\text{O}$ , 0.3 mg/l  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 3.8 mg/l  $\text{H}_3\text{BO}_3$ , 0.18 mg/l  $(\text{NH}_4)_6\text{Mo}_7 \cdot 4\text{H}_2\text{O}$ . For the construction of the cDNA library, three week old plants were irrigated with 200 mM NaCl for 24 hours before harvesting. For the Northern blot analysis plants were irrigated with 250 mM NaCl and the leaves were harvested at different times as indicated in the legend to figure 5. Controls were treated in the same manner but  $\text{H}_2\text{O}$  was supplied to the cultures instead of the NaCl solution.

**Example 2: Yeast strains and culture conditions**

The *Saccharomyces cerevisiae* strain JM26 (*MATa leu 2-3,112 ura 3-1 trp1-1, ade 2-1 his3-11,15 can 1-100, ena 1-4::HIS3, nha1::TRP1*) provided by J.M. Mulet (Universidad Politécnica de Valencia, Instituto de Biología Molecular y Celular de Plantas) was used for the screening of the sugar beet cDNA library and characterization of the *CKA2* cDNA clone. Strain JM26 is a derivative of W303.1A (Wallis *et al.* 1989) with null mutations of the genes *ENA1-4* and *NHA1*, encoding a  $\text{Na}^+$ -pumping ATPase and a  $\text{Na}^+/\text{H}^+$  antiporter, respectively, responsible for most of the yeast sodium extrusion (Garcia de Blas *et al.* 1993, Bañuelos *et al.* 1998). The CK2 temperature sensitive mutant strain YDH8 (*MATa cka1-Δ1::HIS 3 cka2-Δ1::TRP1 ade2-101<sup>ochre</sup> his3-Δ200 leu2-Δ1 lys2-801<sup>amber</sup> trp1-Δ1 ura 3-52* [pDH8: *LEU2 cka2-8*]) was a kind gift of Dr. C.V.C. Glover, University of Georgia (Hanna *et al.* 1995).

Yeast were grown in either minimal synthetic glucose medium (SD) or rich medium (YPD). SD medium contained 2% glucose, 0.7% yeast nitrogen base without amino acids and 50 mM succinic acid, adjusted to pH 5 with Tris, plus the required amino acids [100 μg/ml leucine, 30 μg/ml adenine, 100 μg/ml methionine] as indicated. YPD medium contained 1% yeast extract, 2% Bacto peptone and 2% glucose. Media were supplemented with NaCl as indicated. Solid media contained 2% bacteriological-grade agar.

**Example 3: Construction of a sugar beet cDNA library induced by salt stress**

Directional cDNAs were synthesized (cDNA synthesis kit, Stratagene) using poly(A)<sup>+</sup> RNA prepared from leaves of salt-treated sugar beet plants. cDNAs were ligated into phage λPG15 vector and packaged using a Gigapack III gold packaging extract (Stratagene).

- 5 This phage has inserted the excisable expression plasmid pYPGE15 (*URA3* as a selection marker) that is usable directly for both *Escherichia coli* and yeast complementation (Brunelli and Pall, 1993). A plasmid cDNA library was recovered from λPG15 by the *cre-lox* recombinase system (Brunelli and Pall, 1993).

10 **Example 4: screening and isolation of cDNA clones conferring salt tolerance to yeast**

- To screen for sugar beet cDNAs which increase salt tolerance in yeast, the cDNA library constructed in pYPGE15 was used to transform the yeast mutant strain JM26 by the LiCl method (Gietz *et al.* 1992). Transformants selected on SD plates with leucine and adenine  
15 by uracil prototrophy were pooled and replated on screening medium (SD with leucine, adenine and methionine supplemented with 0.15 M NaCl) at a density of  $2 \times 10^5$  cells per plate (12x12 cm). Methionine was added to the selective medium to avoid selection of the *HAL2*-like homologues already found in *Arabidopsis* (Quintero *et al.* 1996, Gil-Mascarell *et al.* 1999). Alternatively, for the selection of Li<sup>+</sup> resistant yeast cells, the transformants  
20 were replated on screening medium (SD with leucine and adenine supplemented with 20 mM LiCl). The putative positive clones were rescreened on the same NaCl or LiCl medium.

- One of the confirmed NaCl tolerant clones, clone 154, was selected for further characterization. Plasmid DNA was isolated from the 154 yeast cells (pYPGE15+154) and  
25 reintroduced into JM26 to confirm that it conferred salt tolerance. Selection against the *URA3*-marked plasmid using fluorotic acid restored the salt sensitivity of the yeast cells. The insert of pYPGE15+154 was directly sequenced by the dye-primer cycle sequencing method using a DNA sequencer (Model ABI 377, PE Biosystems). After identifying the insert as subunit alpha of CK2 (see below) it was renamed as pYPGE15+BvCKA2.

30

**Example 5: Cloning of the yeast CKA2 gene**

- The *Saccharomyces cerevisiae* CKA2 gene was PCR-isolated from genomic DNA. The amplification was performed with Pwo DNA polymerase (Roche Molecular Biochemicals) and the primers were designed according to the sequence of the genomic clone  
35 (GenBank Accession number: M33759). The sequence of the primers (*Eco* RI-*Xho* I sites underlined) is the following:

Forward primer 5'-ATGGATTAGAATTCTCATAGAGTTGTAAGGTCTCAGGG-3' (SEQ ID NO 11) and reverse primer 5'-CCTCAGTTCTCGAGTTTATAAATGGAAATCAGTGGTGG-3' (SEQ ID NO 12).

The 1.1 kb PCR-amplified product was *Eco* RI- *Xho* I digested and directionally cloned into the yeast expression vector pYPGE15. This construct, named pYPGE15+ScCKA2, was used to transform the yeast strain JM26 (Gietz *et al.* 1992). Sequence analysis confirmed that the insert contained in the pYPGE15 plasmid was the yeast gene *CKA2*.

#### Example 6: Complementation studies of the *CKA2* yeast mutant

As casein kinase 2 catalytic subunit genes (*CKA1* and *CKA2*) are essential for yeast viability, the *ck2* mutant strain YDH8 (*cka1 cka2*) carries the centromeric plasmid pDH8 (*LEU2* marker) coding for a temperature sensitive allele of the catalytic subunit (*cka2-8*). This construct allows cells to grow at 25°C but not at the restrictive temperature of 37°C (Hanna *et al.* 1995). Plasmid pYPGE15+*BvCKA2* was introduced into the YDH8 strain by transformation. Afterwards, plasmid pDH8 was removed from YDH8 by growing cells in rich medium to allow plasmid loss and selecting for leucine auxotrophy. Growth of yeast cells at 25 and 37 °C was investigated.

#### Example 7: Salt tolerance tests and measurements of intracellular ion concentrations

Yeast cultures were pregrown in liquid SD medium with leucine and adenine. Aliquots of saturated cultures were diluted (1:10) and spotted with an 8 x 6 stainless steel replica plater (SIGMA St. Louis, Mo.) on plates containing the indicated concentrations of salts.

For measurements of intracellular ion concentrations 10 ml of yeast culture grown to exponential phase in SD plus leucine, adenine, methionine and 75 mM NaCl (absorbance of 0.7 at 660 nm measured with Spectronic 20D: Milton Roy, Rochester N.Y.) were centrifuged, washed three times by resuspension in ice-cold 10 mM MgCl<sub>2</sub> and finally resuspended in 1 ml of 10 mM MgCl<sub>2</sub>. Cell concentration was determined by the absorbance at 660 nm, and intracellular ions were extracted by addition of concentrated HCl to a 0.1 M final concentration. After removal of cell debris by centrifugation, K<sup>+</sup> and Na<sup>+</sup> concentrations in the supernatant were determined by atomic absorption spectrometer (Varian) in flame emission mode. Intracellular water was estimated as previously described (Gaxiola *et al.* 1992).



**Example 8: Southern and Northern blot analyses**

Genomic DNA was prepared from leaves of 3 week old sugar beet according to Rogers and Bendich (1994). Five µg of DNA were digested with appropriate restriction enzymes, electrophoresed in 0.8 % agarose gel and blotted onto a nylon membrane filter (Hybond N+, Amersham Life Science). The membrane filter was hybridized with two different <sup>32</sup>P-labeled DNA probes. One of them corresponded to a 887 bp PCR amplified fragment (forward primer, 5'-CGAAGCTCTCACTGTTCAATGG-3' (SEQ ID NO 13) and reverse primer, 5'-GGATGTGCCATTG CTTCTTTTGC-3' (SEQ ID NO 14)) including in the coding region of *BvCKA2* cDNA. A second more specific fragment of 323 bp (forward primer, 5'-CTCTCAAGTTAGAGCTGCAGA-3' (SEQ ID NO 15) and reverse primer, 5'-GCTATTAGCAAACCTATATTAAGTG-3' (SEQ ID NO 16)) includes the 3' non-coding region of *BvCKA2* cDNA. Hybridization and washes were carried out under high-stringency conditions (65° C) according to Church and Gilbert (1984).

For Northern blot analysis total RNA was isolated from control or Na<sup>+</sup>-treated sugar beet leaves as described by Davis *et al.* (1998). Thirty µg of total RNA were separated on a 1% agarose gel containing 2.2% formaldehyde and blotted onto a nylon membrane filter (Hybond N, Amersham Life Science). Hybridization was carried out with the 3'UTR specific probe of 323 bp described above for the Southern blotting. The filter was washed twice with SSC 4X, 0.1 % SDS for 5 min and twice with SSC 0.4X, 0.1 SDS for 5 min at 65° C. The same filter was rehybridized with a 1.9 *Eco* RI fragment comprising the α<sub>3</sub> tubulin gene of *Arabidopsis* (Ludwig *et al.* 1987). Hybridization and wash temperatures were reduced to 55° C for this heterologous probe.

**Example 9: Rice transformation with the sugar beet genes**

Expression of sugar beet genes involved in salt tolerance in yeast in rice mediating stress tolerance in rice.

To investigate the stress tolerance activation of the sugar beet stress tolerance genes in monocots, the aforementioned genes (SEQ ID NO. 1 – 5 ), operably linked to a promoter, are each transformed to rice using the standard transformation procedures well known to the persons skilled in the art and outlined in the following paragraph. After several time periods ranging from 1 day to 1 or more weeks, the seedling is checked for the expression of the transformed gene. This is done by growing the seedlings in organogenesis medium, and checking the presence of the DNA or mRNA by PCR or reverse PCR. After the confirmation of gene expression the transformed rice plants are checked for the enhanced tolerance to stress situations including salt, drought and cold (see WO97/13843). This is done by growing the transformed rice plants in medium containing

increased amounts of NaCl or LiCl. Also the increased resistance to cold or drought is tested by growing the transformed plants in suboptimal growing temperatures and suboptimal levels of humidity, respectively (WO97/13843).

## 5 Agrobacterium-mediated rice transformation

The sugar beet genes of the present invention can be operably linked to a promoter and cloned into a vector. These vectors can be transformed to *Agrobacterium tumefaciens* strain LBA4404 or C58 by means of electroporation and subsequently transformed bacterial cells can be selected on a solid agar medium containing the appropriate antibiotics.

For demonstration of the expression of the genes of current invention in rice, 309 mature dry seeds of the rice japonica cultivars Nipponbare or Taipei are dehusked, sterilised and germinated on a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D). After incubation in the dark for four weeks, embryogenic, scutellum-derived calli are excised and propagated on the same medium. Selected embryogenic callus is then co-cultivated with *Agrobacterium*. Co-cultivated callus is grown on 2,4-D-containing medium for 4 to 5 weeks in the dark in the presence of a suitable concentration of the appropriate selective agent. During this period, rapidly growing resistant callus islands develop. After transfer of this material to a medium with a reduced concentration of 2,4-D and incubation in the light, the embryogenic potential is released and shoots develop in the next four to five weeks. Shoots are excised from the callus and incubated for one week on an auxin-containing medium from which they can be transferred to the soil. Hardened shoots are grown under high humidity and short days in a phytotron. Seeds can be harvested three to five months after transplanting. The method yields single locus transformants at a rate of over 50 % (Chan *et al.* 1993, Hiei *et al.* 1994).

### Example 10: Transformation of *Arabidopsis* with *Beta vulgaris* CKA2 gene

The tolerance to NaCl of transgenic plants expressing 154cDNA (*BvCKA2*) was studied.

*Arabidopsis thaliana* plants (Ecotype Columbia) were transformed either with the empty plasmid pBI121 (CLONTECH) or pBI121*BvCKA2* expressing the sugar beet CK2 protein kinase catalytic subdomain (SEQ ID Nr. 6). *BvCKA2* cDNA was inserted between the BamHI and Sac I sites of pBI121. The constructs were introduced into *Agrobacterium tumefaciens* C58Rif<sup>R</sup> Rif strain (Van Larebeke *et al.* 1974). This strain was used to transform *Arabidopsis* plants by the floral dipping method.

The effect of NaCl on plant survival was determined *in vitro* and in greenhouse experiments.

In vitro experiments.

Seeds were grown in plates with MS medium plus 100 or 125 mM NaCl. Survival was determined as the number of seedlings that developed cotyledons and/or true leaves.

- 5 Results are shown in figure 7 and 8. These results clearly show that the Arabidopsis plants transfected with the cDNA having a nucleic acid sequence as represented in SEQ ID NO 1, encoding a the *Beta vulgaris* casein kinase  $\alpha$  subunit having an amino acid sequence as represented in SEQ ID NO 6 of the present invention, clearly have an enhanced survival rate in salt stress conditions, when compared to wild type plants.

10

Greenhouse experiments.

Plants were sown on pots containing a mixture of soil and vermiculite (2:1). The plants were grown as previously described (Kanhonou *et al.* 2001). Tolerance to 50 mM NaCl of control plants (Wild type plants and plants transformed with the empty plasmid) and plants overexpressing *BvCKA2* was determined as the percentage of adult plants per sowed plants (Table 5). The dry weight was also determined (Table 5). The data are the mean  $\pm$  SD.

15

TABLE 5.

PLANT	Control Medium		50 mM NaCl	
	% Adult plants/Sowed plants	Shoot dry weight (mg/plant)	% Adult plants/Sowed plants	Shoot dry weight (mg/plant)
Control WT	93 $\pm$ 3	103 $\pm$ 8	46 $\pm$ 5	32 $\pm$ 7
Transgenic Control	89 $\pm$ 6	84 $\pm$ 27	48 $\pm$ 4	33 $\pm$ 10
TBvCKA2,1	90 $\pm$ 3	96 $\pm$ 11	52 $\pm$ 1.5	33 $\pm$ 10
TBvCKA2,2	93 $\pm$ 11	115 $\pm$ 11	58 $\pm$ 2	47 $\pm$ 3
TBvCKA2,3	90 $\pm$ 6	110 $\pm$ 10	62 $\pm$ 4	45 $\pm$ 3

20

**Example 11: The use of *BvCKA2* gene for controlling the flowering independently of the photoperiod.**

CK2 has recently been described as a Quantitative trait loci (QTL) involved in controlling flowering time (Takahashi *et al.* 2001). Specifically CK2 seems to be involved in controlling photoperiod sensitivity.

25

The inventors observed that the transgenic *Arabidopsis* plants as described in example 10 have a delay in the flowering time. This phenotype has a practical use, since growers are interested in controlling the process of flowering independently of the photoperiod. Delays or advances of flowering time is regulated by controlling the level of expression of CK2, or by modulating the activity of the CK2 protein in a plant cell or by using mutants of this protein.

#### Example 12: Functional characterization of BveIF-1A

The *Saccharomyces cerevisiae* yeast strain JM26 (Kanhonou *et al.* 2001) was transformed with either the empty plasmid pYPGE15 (Brunelli and Pall 1993) or 76pYPGE15. Plasmid 76pYPGE15 carries a *Beta vulgaris* cDNA coding for the translation initiation factor e-IF1A. The incorporation of radioactive phenylalanine into yeast proteins was determined in liquid culture as described (Pascual Ahuir *et al.* 2001). The experiment was done in the presence (pYPGE15-300mM NaCl, 76pYPGE15- 300mM NaCl) or absence (pYPGE15, 76pYPGE15) of 300 mM NaCl. Results are shown in Figure 9. These results show clearly in the absence of NaCl the transgenic yeast carrying the BveIF-1A incorporate less phenylalanine compared to the cells transformed with the empty vector. On the contrary, when the yeast cells are put under severe salt stress conditions it is clear that protein synthesis occurs much better in the yeast cells transformed with the BveIF-1A.

20

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## CLAIMS

1. Use of a *Beta vulgaris* nucleic acid for enhancing stress tolerance in a plant comprising expression of said *Beta vulgaris* nucleic acid characterized in that it confers stress tolerance to yeast cells
- 5 2. Use of a *Beta vulgaris* nucleic acid according to claim 1 wherein said yeast cells are derived from the Na<sup>+</sup> sensitive yeast strain JM26.
3. Use of a *Beta vulgaris* nucleic acid according to claim 1 or 2 for enhancing osmotic or oxidative stress tolerance to a plant wherein said *Beta vulgaris* nucleic acid confers osmotic or oxidative stress tolerance to said yeast cells.
- 10 4. Use of a *Beta vulgaris* nucleic acid for enhancing osmotic or oxidative stress tolerance in a plant according to any of claims 1 to 3 wherein said *Beta vulgaris* nucleic acid is chosen from:
  - (a) a nucleic acid comprising a DNA sequence as given in any of SEQ ID NOs 1 to 5 or the complement thereof,
  - 15 (b) a nucleic acid comprising the RNA sequence corresponding to any of SEQ ID NOs 1 to 5 or the complement thereof,
  - (c) a nucleic acid specifically hybridizing to the nucleic acid of (a) or (b) under high stringency conditions,
  - (d) a nucleic acid encoding a protein with an amino acid sequence which is at least 20 93% identical to the amino acid sequence as given in SEQ ID NO 6,
  - (e) a nucleic acid encoding a protein with an amino acid sequence which is at least 80% identical to the amino acid sequence as given in SEQ ID NO 7,
  - (f) a nucleic acid encoding a protein with an amino acid sequence which is at least 89% identical to the amino acid sequence as given in SEQ ID NO 8,
  - 25 (g) a nucleic acid encoding a protein with an amino acid sequence which is at least 75% identical to the amino acid sequence as given in SEQ ID NO 9,
  - (h) a nucleic acid encoding a protein with an amino acid sequence which is at least 65% identical to the amino acid sequence as given in SEQ ID NO 10,
  - (i) a nucleic acid encoding a protein comprising the amino acid sequence as given in 30 any of SEQ ID NOs 6 to 10,
  - (j) a nucleic acid encoding an immunologically active and/or functional fragment of a protein encoded by a DNA sequence as given in any of SEQ ID NOs 1 to 5,
  - (k) a nucleic acid which is degenerated to a nucleic acid as given in any of SEQ ID NOs 1 to 5, or which is degenerated to a nucleic acid as defined in any of (a) to (j) 35 as a result of the genetic code,

- (l) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs 6 to 10, or which is diverging from a nucleic acid as defined in any of (a) to (j) as a result of differences in codon usage between organisms,
- (m) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs 6 to 10, or which is diverging from a nucleic acid as defined in any of (a) to (j) as a result of differences between alleles, and
- (n) a nucleic acid as defined in any one of (a) to (m) characterized in that said nucleic acid is DNA, cDNA, genomic DNA or synthetic DNA.
5. A method for enhancing stress tolerance in a plant comprising the expression or altering the expression of any nucleic acid as defined in claim 4 in cells, tissues or parts of said plant.
6. A method for enhancing stress tolerance in a plant comprising the expression or altering the expression of a nucleic acid encoding a casein kinase alpha subunit in cells, tissues or parts of said plant.
7. A method according to claim 6 wherein said nucleic acid encodes a casein kinase alpha subunit as defined in claim 4, or a homologue, an orthologue or a paralogue thereof.
8. A method for enhancing stress tolerance in a plant comprising the expression or altering the expression of a nucleic acid encoding a dihydroorotase in cells, tissues or parts of said plant.
9. A method according to claim 8 wherein said nucleic acid encodes a dihydroorotase as defined in claim 4, or a homologue, an orthologue or a paralogue thereof.
10. A method for enhancing stress tolerance in a plant comprising the expression or altering the expression of a nucleic acid encoding a translation initiation factor 1A in cells, tissues or parts of said plant.
11. A method according to claim 10 wherein said nucleic acid encodes a translation initiation factor 1A as defined in claim 4, or a homologue, an orthologue or a paralogue thereof.
12. A method for enhancing stress tolerance in a plant comprising the expression or altering the expression of a nucleic acid as represented by SEQ ID NO 4 or 5, or a homologue, an orthologue or a paralogue thereof.
13. A method according to any of claims 5 to 12 wherein said expression of said nucleic acid occurs under the control of a promoter.
14. A method according to any of claims 5 to 13 wherein said stress is osmotic stress.

15. A method according to claim 14 wherein said osmotic stress is caused by salt.
16. A method according to claim 14 wherein said osmotic stress is caused by drought.
17. A method according to claim 14 wherein said osmotic stress is caused by frost.
18. A method according to claim 14 wherein said osmotic stress is caused by cold.
- 5 19. A method according to any of claims 5 to 18 wherein said method leads to an increase in yield.
20. An isolated nucleic acid encoding a protein for enhancing osmotic or oxidative stress tolerance to a plant, or an immunologically active and/or functional fragment of such a protein selected from one of the following:
- 10 (a) a nucleic acid comprising a DNA sequence as given in any of SEQ ID NOs 1 to 5 or the complement thereof,
- (b) a nucleic acid comprising the RNA sequence corresponding to any of SEQ ID NOs 1 to 5 or the complement thereof,
- 15 (c) a nucleic acid specifically hybridizing to the nucleic acid of (a) or (b) under high stringency conditions,
- (d) a nucleic acid encoding a protein with an amino acid sequence which is at least 93% identical to the amino acid sequence as given in SEQ ID NO 6,
- (e) a nucleic acid encoding a protein with an amino acid sequence which is at least 80% identical to the amino acid sequence as given in SEQ ID NO 7,
- 20 (f) a nucleic acid encoding a protein with an amino acid sequence which is at least 89% identical to the amino acid sequence as given in SEQ ID NO 8,
- (g) a nucleic acid encoding a protein with an amino acid sequence which is at least 75% identical to the amino acid sequence as given in SEQ ID NO 9,
- (h) a nucleic acid encoding a protein with an amino acid sequence which is at least 65% identical to the amino acid sequence as given in SEQ ID NO 10,
- 25 (i) a nucleic acid encoding a protein comprising the amino acid sequence as given in any of SEQ ID NOs 6 to 10,
- (j) a nucleic acid encoding an immunologically active and/or functional fragment of a protein encoded by a DNA sequence as given in any of SEQ ID NOs 1 to 5,
- 30 (k) a nucleic acid which is degenerated to a nucleic acid as given in any of SEQ ID NOs 1 to 5, or which is degenerated to a nucleic acid as defined in any of (a) to (j) as a result of the genetic code,
- (l) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs 6 to 10, or which is diverging from a nucleic acid as defined in any of (a) to (j) as a result of differences in codon usage between organisms,
- 35

- (m) a nucleic acid which is diverging from a nucleic acid encoding a protein as given in any of SEQ ID NOs 6 to 10, or which is diverging from a nucleic acid as defined in any of (a) to (j) as a result of differences between alleles, and
- (n) a nucleic acid as defined in any one of (a) to (m) characterized in that said nucleic acid is DNA, cDNA, genomic DNA or synthetic DNA.

5

21. A nucleic acid molecule of at least 15 contiguous nucleotides in length specifically hybridizing with a nucleic acid of claim 20.

22. A nucleic acid molecule of at least 15 contiguous nucleotides in length specifically amplifying a nucleic acid of claim 20.

10 23. A vector comprising a nucleic acid sequence according to claim 20.

24. A vector according to claim 23 which is an expression vector wherein said nucleic acid sequence is operably linked to one or more control sequences allowing the expression of said sequence in prokaryotic and/or eukaryotic host cells.

15

25. A host cell containing a nucleic acid molecule according to claim 20 or a vector according to claim 23 or 24.

26. A host cell according to claim 25, wherein the host cell is a bacterial, insect, fungal, yeast, plant or animal cell.

27. An isolated polypeptide encodable by a nucleic acid of claim 20, or a homologue or a derivative thereof, or an immunologically active and/or functional fragment thereof.

20

28. A polypeptide of claim 27 having an amino acid sequence as given in any of SEQ ID NOs 6 to 10, or a homologue or a derivative thereof, or an immunologically active and/or functional fragment thereof.

25

29. A method for producing a polypeptide of claim 27 or 28 comprising culturing a host cell of claim 25 or 26 under conditions allowing the expression of the polypeptide and recovering the produced polypeptide from the culture.

30. An antibody specifically recognizing a polypeptide of claim 27 or 28 or a specific epitope of said polypeptide.

30

31. A method for the production of altered plant cells, plant tissues or plants comprising the introduction of a polypeptide of claim 27 or 28 directly into said plant cell or tissue or in an organ of said plant.

32. A method for effecting the expression of a polypeptide of claim 27 or 28 comprising the introduction of a nucleic acid molecule of claim 20 operably linked to one or more control sequences or a vector of claim 23 or 24 stably into the genome of a plant cell.

33. A method for the production of transgenic plant cells, plant tissues or plants comprising the introduction of a nucleic acid of claim 20 in an expressible format or a vector of claim 23 or 24 in said plant cell, plant tissue or plant.
34. A method for enhancing osmotic or oxidative stress tolerance in a plant cell, tissue or  
5 plant comprising the introduction of any nucleic acid as identified in any of claims 5 to 13 into said plant cell, tissue or organ of said plant
35. A method according to any of claims 32 to 34 further comprising regenerating a plant from said plant cell.
36. A transgenic plant cell comprising a nucleic acid of claim 20 which is operably linked to  
10 regulatory elements allowing transcription and/or expression of said nucleic acid in plant cells or a transgenic plant cell obtainable by a method of claim 33.
37. A transgenic plant cell of claim 36 wherein said nucleic acid of claim 20 is stably integrated into the genome of said plant cell.
38. A transgenic plant or plant tissue comprising plant cells of claim 36 or 37 or a  
15 transgenic plant obtainable by the method of claim 35.
39. A transgenic plant of claim 38 which displays increased tolerance to stress, preferably osmotic or oxidative stress, compared to the corresponding wild type plant.
40. A harvestable part of a plant of claim 38 or 39.
41. The harvestable part of a plant of claim 40 which is selected from the group consisting  
20 of seeds, leaves, fruits, stem cultures, rhizomes and bulbs.
42. The progeny derived from any of the plants or plant parts of any of claims 38 to 41.
43. A method for identifying and obtaining proteins interacting with a polypeptide of claim 27 or 28 comprising a screening assay wherein a polypeptide of claim 27 or 28 is used.
- 25 44. The method of claim 43 comprising a two-hybrid screening assay wherein a polypeptide of claim 27 or 28 as a bait and a cDNA library as prey are used.
45. A method for modulating the interaction between a polypeptide of claim 27 or 28 and interacting protein partners obtainable by a method according to claim 43 or 44.
- 30 46. A method for identifying and obtaining compounds interacting with a polypeptide of claim 27 or 28 comprising the steps of:  
(a) providing a two-hybrid system wherein a polypeptide of claim 27 or 28 and an interacting protein partner obtainable by a method according to claim 43 and 44 are expressed,

- (b) interacting said compound with the complex formed by the expressed polypeptides as defined in a), and,
- (c) performing measurement of interaction of said compound with said polypeptide or the complex formed by the expressed polypeptides as defined in (a).
- 5 47. A method for identifying compounds or mixtures of compounds which specifically bind to a polypeptide of claim 27 or 28, comprising the steps of:
- (a) combining a polypeptide of claim 27 or 28 with said compound or mixtures of compounds under conditions suitable to allow complex formation, and,
- 10 (b) detecting complex formation, wherein the presence of a complex identifies a compound or mixture of compounds which specifically binds said polypeptide.
48. Use of a compound or mixture of compounds identified by means of a method of claim 46 or 47 as a factor that enhances stress tolerance in plants.
49. Use of a nucleic acid of claim 20, a vector of claim 23 or 24, a polypeptide of claim 27 or 28 for increasing yield.
- 15 50. Use of a nucleic acid molecule of claim 20, a vector of claim 23 or 24, a polypeptide of claim 27 or 28 for stimulating plant growth, which can be in any part of that plant, such as root, leaf or seed.
51. Diagnostic composition comprising at least a nucleic acid of claim 20, a vector of claim 23 or 24, a polypeptide of claim 27 or 28 or an antibody of claim 30.
- 20 52. Use of a plant obtainable by a method of claim 31 or 32 or the plant of claim 38 or 39 for culturing on soil with a salt content of more than 1mM salt ions.
53. A method for controlling the process of flowering of a plant comprising the expression or altering the expression of a nucleic acid encoding a casein kinase alpha subunit represented by SEQ ID NO 6 in cells, tissues or parts of said plant.
- 25 54. Use of a nucleic acid encoding a casein kinase alpha subunit represented by SEQ ID NO 6 for controlling the process of flowering of a plant.



**FIGURE 1**

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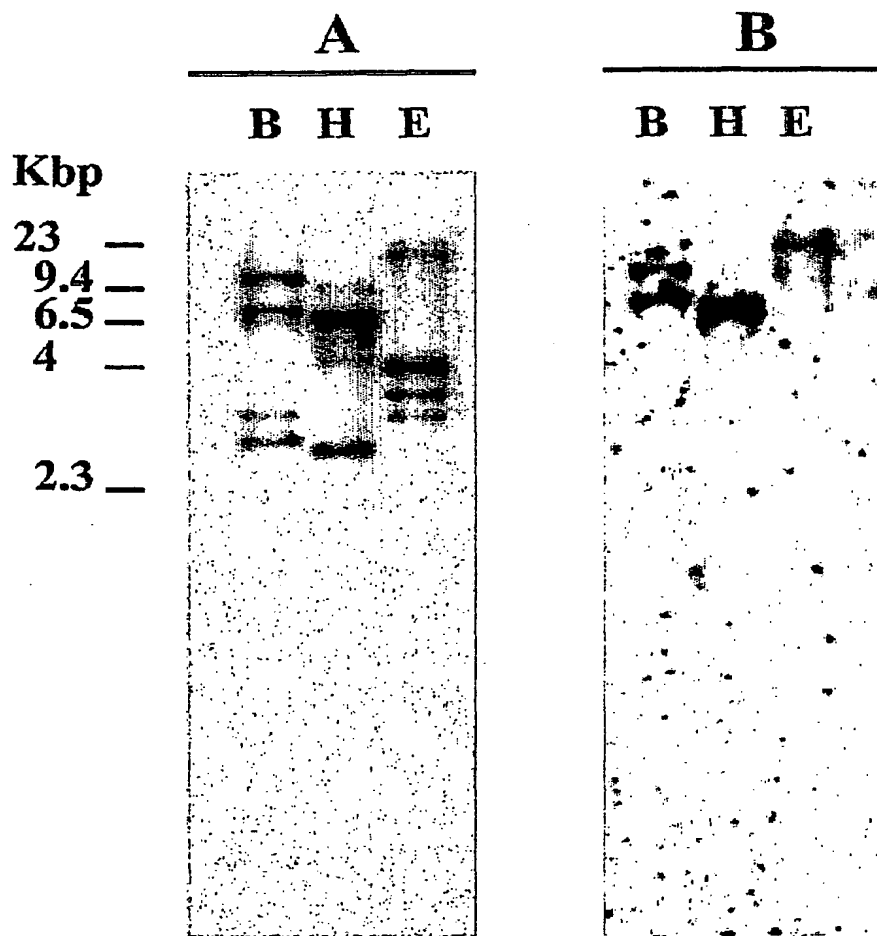


FIGURE 2

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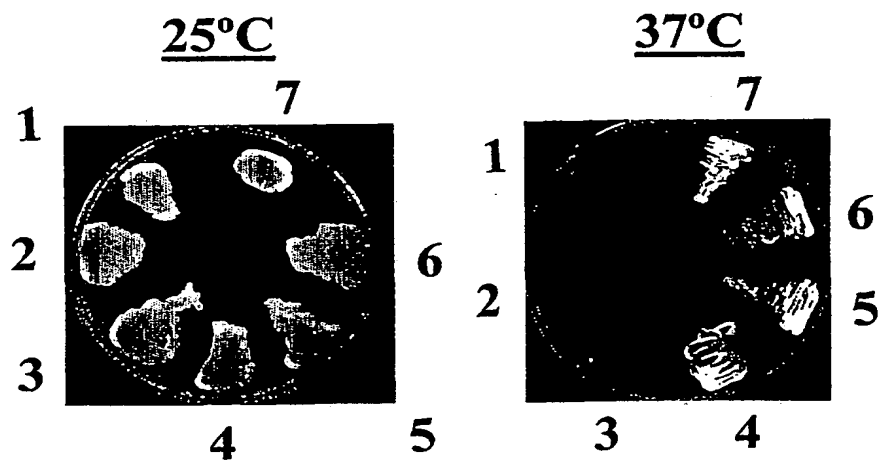


FIGURE 3

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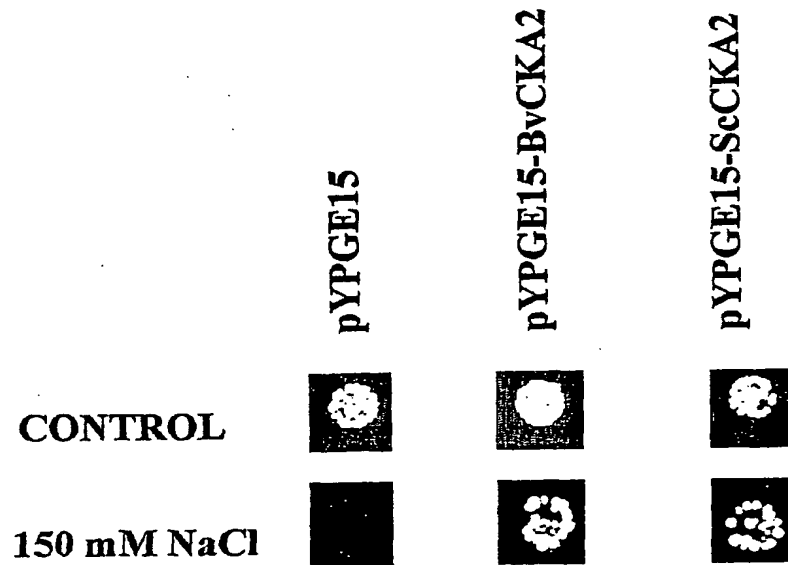


FIGURE 4

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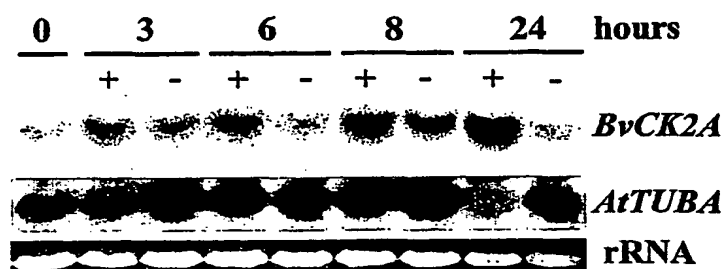


FIGURE 5

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Figure 6 :

SEQ ID NO 1 : DNA sequence of the red beet gene clone 154 BvCK2 (casein kinase  $\alpha$  catalytic subunit)

```
GAATTCGGCACGAGGAAATTTGAGATTGTCGGGTTCACCTCTTCACACTACTAACTTTTT
TCCATCTTCTCTCTCACTCGTCTTACTGTGCGCACTCCTTGCTCTCCGTGCACCGGTGGCGC
ATCCTCCTATCCTGCGCCCATCAACCCTAAATTTGCGCCGCGCTAATTTTCGAGATCTCCGCC
GACGCAAATTTCTCCGATGTCGAAGTCGCGAGTTTACGCCGACGTTAATGTTCTCCGACCTCG
AGAGTACTGGGATTACGAAGCTCTCACTGTTCAATGGGGTGATCAAGATGACTATGAAGTCG
TGCGGAAGATTGGAAGGGGGAAATACAGTGAAGTTTTTGAAGGATTAAACGTCAATAGTAAT
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SEQ ID NO 2 : DNA sequence of the red beet gene clone 35 BvDHO (dihydroorotase)

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SEQ ID NO 3 : DNA sequence of the red beet gene clone 76 BvelF-1A  
(translation initiation Factor 1A)

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SEQ ID NO 4 : DNA sequence of the red beet gene clone 120 Bv120 (putative  
protein)

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SEQ ID NO 5 : DNA sequence of the red beet gene clone 20Li Bv20Li (unknown protein)

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SEQ ID NO 6 : amino acid sequence of the red beet gene clone 154 BvCK2  
 (casein kinase  $\alpha$  catalytic subunit)

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 GLNVNSNERC VIKILKPVKK KIKREIKIL QNLGGPNVI KLLDIVRDQH  
 SKTPSLVFEF VNSTDFKVLY PTLSDYDIRY YIYELLKALD FCHSQGIMHR  
 DVKPHNV MID HELRKLRLID WGLAEFYHPG KEYNVRVASR YFKGPELLVD  
 LQDYDYS LDM WSLGCMFAGM IFRKEPFFYG HDNHDQLVKI AKVLGTDELN  
 AYLNKYHLEL DPQLDALVGR HSRKPWSRFV NPDNQHLVSP EAIDFLDKLL  
 RYDHQDRLTA KEAMAHYPYFS QVRAAESSRM RTQ\*

SEQ ID NO 7 : amino acid sequence of the red beet gene clone 35  
 BvDHO(dihydroorotase)

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 KVFIESVLRP LIQKLPQLKV VMEHITTADA VKFIESCNGG NVAATVTPQH  
 LVLNRNSLFQ GGLQPHNYCL PVLKREIHRQ ALVSAVTS GS KQYFLGTDSA  
 PHERRRKECS CGCAGIYNP VALSLYAKVF EEAGALDKLE AFTSFNGPDF  
 YGLPRNTSKI KLKKEPWKVL ERIPFPSGEI IPMFAGQMLD WKPSF\*

SEQ ID NO 8 : amino acid sequence of the red beet gene clone 76 BveIF-1A  
 (translation initiation Factor 1A)

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 RLLKAYGELP DNIRLNEGVA NLDEEDDGA DDYIEFEDED IDKI\*



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SEQ ID NO 9 : amino acid sequence of the red beet gene clone 120 Bv120  
(putative protein)

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IGTTIGPKGT VETDKDSFNY QKALQDKKFE QEAHKLWFRM RNEVVAELQE  
KGFDVE\*

SEQ ID NO 10 : amino acid sequence of the red beet gene clone 20Li Bv20Li  
(unknown protein)

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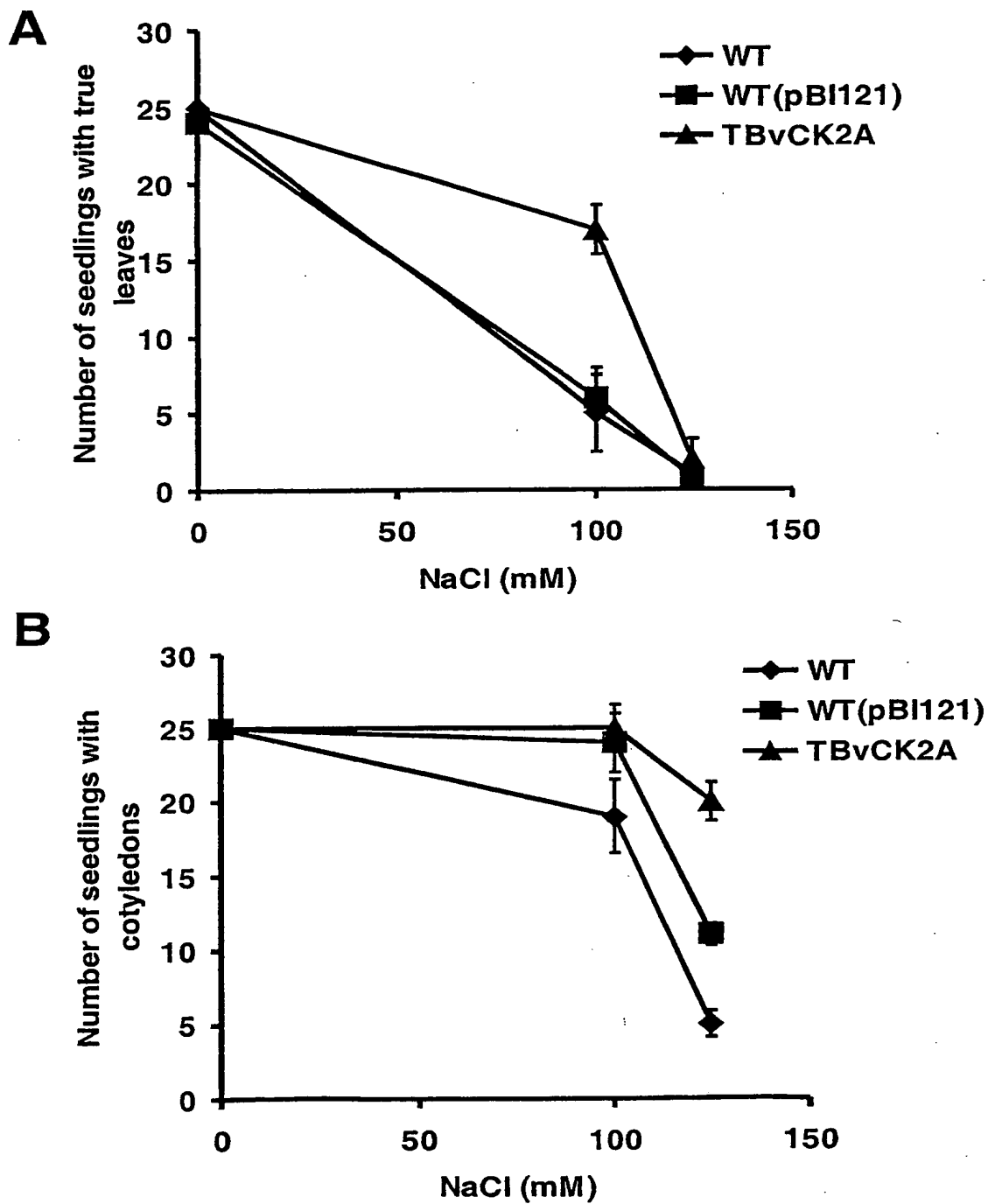


FIGURE 7

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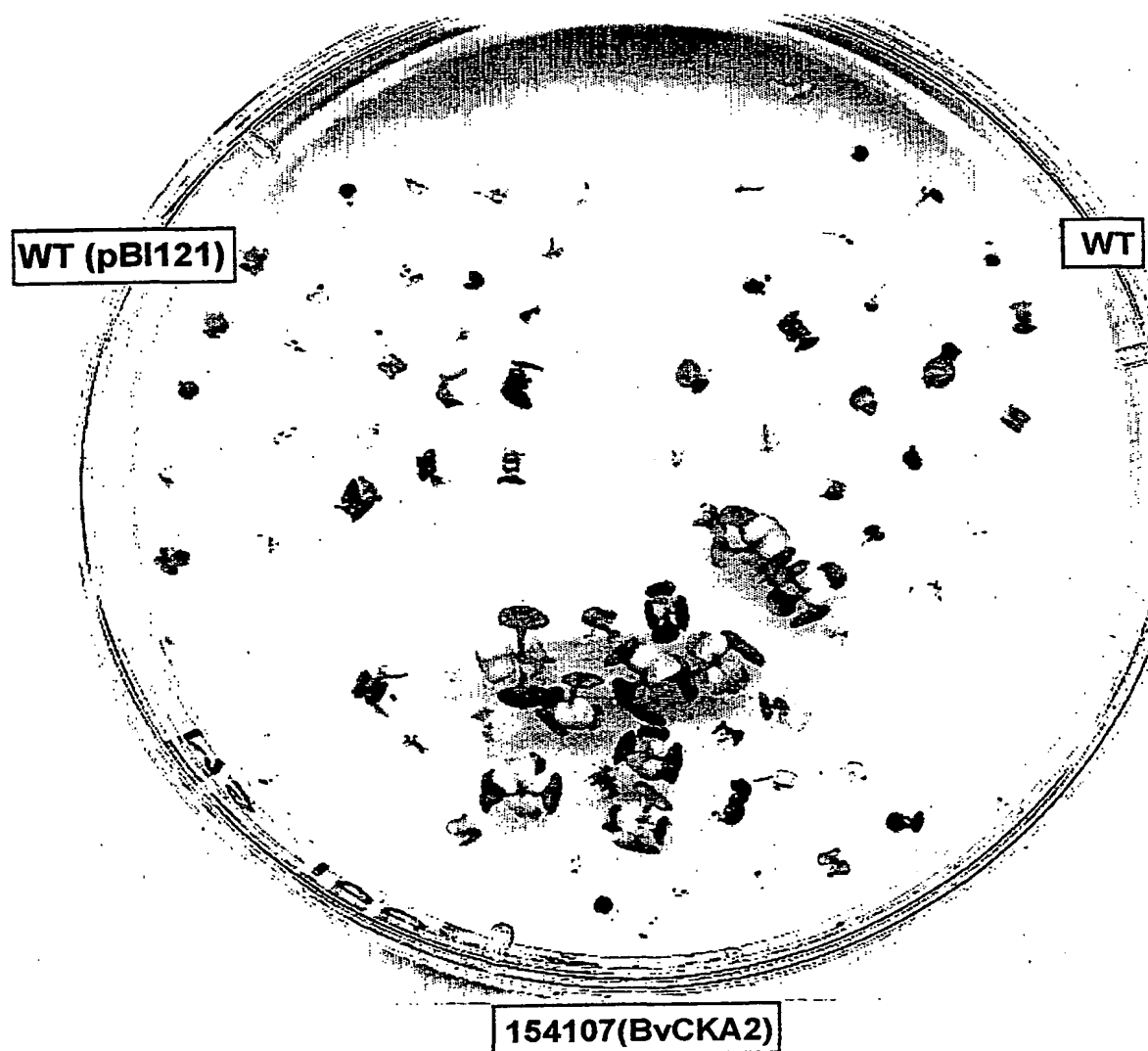
**MS+100 mM NaCl after 12 days culture**

FIGURE 8

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### Quantification of radioactive phenylalanin incorporation into proteins

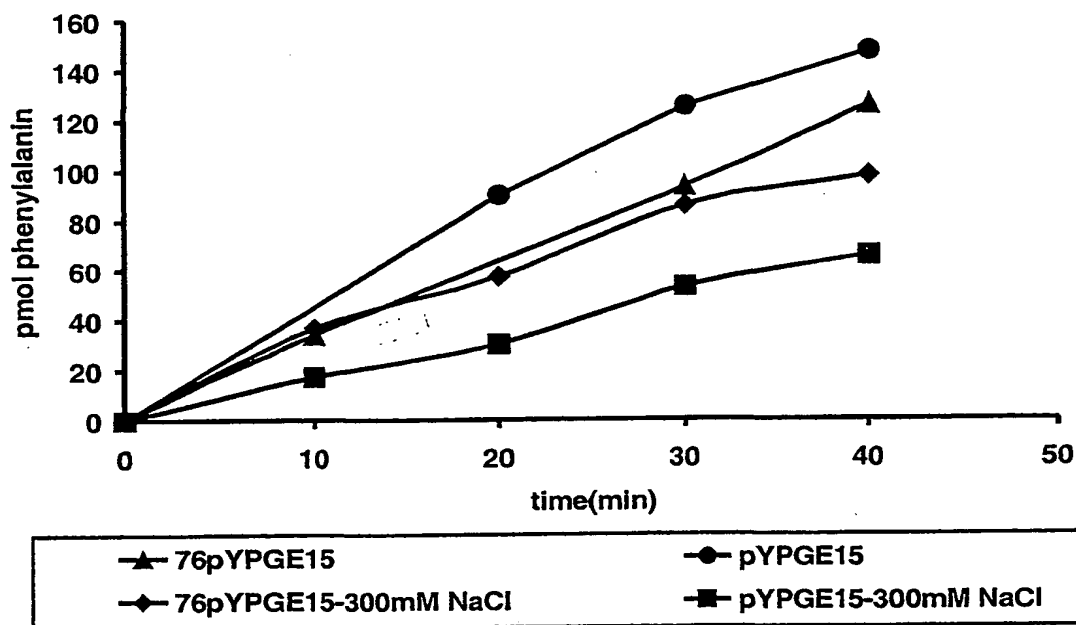


FIGURE 9

## SEQUENCE LISTING

<110> CropDesign N.V.

<120> Red beet genes involved in stress tolerance

<130> CROP-017-PCT

<150> EP 00870319.1

<151> 2000-12-22

<150> US 60/271,656

<151> 2001-02-26

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Ser Thr Asp Phe Lys Val Leu Tyr Pro Thr Leu Ser Asp Tyr Asp Ile  
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14/415, C12N 9/86, 15/55, 1/19, 1/21, 5/10, C07K 16/16,  
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AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
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GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
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(84) Designated States (*regional*): ARIPO patent (GH, GM,  
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European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
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For two-letter codes and other abbreviations, refer to the "Guid-  
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ning of each regular issue of the PCT Gazette.

(54) Title: SUGAR BEET GENES INVOLVED IN STRESS TOLERANCE

(57) Abstract: The present invention relates to isolated genes originating from Beta vulgaris, sugar beet, that are involved in responses to stress situations. The genes were isolated from a sugar beet cDNA library screened in a functional selection procedure with transformed yeast cells that were able to grow in selection medium with high salt concentrations. Subsequently these genes were sequenced and further characterized. One of the genes is a sugar beet casein kinase a \$(g)a\$-subunit, one is a sugar beet dihydroorotase, one is a sugar beet translation initiation factor 1A and two others are of a unknown protein type. All of these isolated plant genes were functional as stress tolerance enhancers in yeast cells and are therefore useful to confer stress tolerance to an organism when transfected herein. More particularly, these genes can be used to render crops resistant to stress situations like osmotic stress caused by salt, drought, cold or frost.

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## INTERNATIONAL SEARCH REPORT

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/12 C12N15/29 C12N15/54 C12N15/63 C12N15/80  
 C12N15/82 C12N15/85 C12N15/81 C07K14/415 C12N9/86  
 C12N15/55 C12N1/19 C12N1/21 C12N5/10 C07K16/16

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## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K C12Q A01H G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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MEDLINE, EPO-Internal, BIOSIS, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>G. PERACCHIA ET AL: "CHARACTERISATION, SUBCELLULAR LOCALIZATION AND NUCLEAR TARGETING OF CASEIN KINASE 2 FROM ZEA MAYS"</p> <p>PLANT MOLECULAR BIOLOGY, vol. 10, 1999, pages 199-211, XP002181563 abstract</p> <p>page 200, column 1, paragraph 3</p> <p>page 200, column 2, paragraph 2 - paragraph 3; figure 1</p> <p style="text-align: center;">--- -/-</p>	1-3,6-10

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\*Z\* document member of the same patent family

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Name and mailing address of the ISA

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Authorized officer

Keller, Y

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/15093

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C12Q1/68

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## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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X	MI-JEONG ET AL: "Isolation and characterisation of the gene encoding glyceraldehyde-3-phosphate dehydrogenase" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 278, no. 1, 2000, pages 192-196, XP000216272	1,2
Y	abstract page 193, column 1, paragraph 2 -column 2, paragraph 2 page 195, column 1, paragraph 2 --- -/--	3,6-10



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Fax (+31-70) 340-3016

Authorized officer

Keller, Y

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/15093

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAI LAN PIAO ET AL: "An Arabidopsis GSK3/shaggy-like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and Absciscic Acid" PLANT PHYSIOLOGY, vol. 119, April 1999 (1999-04), pages 1527-1534, XP002181564	1,2
Y	abstract page 1528, column 1, paragraph 3 -column 2, paragraph 3 figures 1,7,9 page 1531, column 1, paragraph 3 -page 1532, column 2, paragraph 1	3,6-10
X	TATJANA KLEINOW ET AL: "Functional identification of an Arabidopsis snf4 ortholog by screening for heterologous multicopy suppressor of snf4 deficiency in yeast" THE PLANT JOURNAL , vol. 23, no. 1, 2000, pages 115-122, XP002181565 abstract page 115, column 1, paragraph 1 -column 2, paragraph 2 table 1 figure 2 page 119, column 2, paragraph 2 -page 1120, column 1, paragraph 1 page 120, column 2, paragraph 2 -page 121, column 1, paragraph 1	1,2
X	DATABASE EMBL 'Online! Acc. No Y11526, 2 November 1998 (1998-11-02) XP002181566 the whole document	6-10
T	I. WINICOV: "New molecular approaches to improving salt tolerance in crop plants" ANNALS OF BOTANY, vol. 82, 1998, pages 703-710, XP001007288	

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15/29, 15/54, 15/63, 15/80, 15/82, 15/85, 15/81, C07K  
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16/40, A01H 15/00, 5/10, G01N 33/50, 33/53, C12Q 1/68(21) International Application Number: **PCT/EP01/15093**(22) International Filing Date:  
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(72) Inventors; and

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Asturias, 22, 2, E-46023 Valencia (ES). **ROS PALAU**,  
Roque [ES/ES]; Somells, 20, 13, E-46006 Valencia (ES).(74) Agents: **DE CLERCQ**, Ann et al.; De Clercq, Brants &  
Partners, E. Gevaertdreef 10 a, B-9830 Sint-Martens-Latem  
(BE).(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
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LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,  
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU,  
ZA, ZW.(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
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GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
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see PCT Gazette No. 08/2003 of 20 February 2003, Sec-  
tion IIFor two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.(54) Title: **SUGAR BEET GENES INVOLVED IN STRESS TOLERANCE**(57) Abstract: The present invention relates to isolated genes originating from *Beta vulgaris*, sugar beet, that are involved in responses to stress situations. The genes were isolated from a sugar beet cDNA library screened in a functional selection procedure with transformed yeast cells that were able to grow in selection medium with high salt concentrations. Subsequently these genes were sequenced and further characterized. One of the genes is a sugar beet casein kinase a \$(g)a\$-subunit, one is a sugar beet dihydroorotase, one is a sugar beet translation initiation factor 1A and two others are of a unknown protein type. All of these isolated plant genes were functional as stress tolerance enhancers in yeast cells and are therefore useful to confer stress tolerance to an organism when transfected herein. More particularly, these genes can be used to render crops resistant to stress situations like osmotic stress caused by salt, drought, cold or frost.

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C12N9/12	C12N15/29	C12N15/54	C12N15/63	C12N15/80
	C12N15/82	C12N15/85	C12N15/81	C07K14/415	C12N9/86
	C12N15/55	C12N1/19	C12N1/21	C12N5/10	C07K16/16

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K C12Q A01H G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Keller, Y

## INTERNATIONAL SEARCH REPORT

International Application No

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K16/40 A01H15/00 A01H5/10 G01N33/50 G01N33/53  
C12Q1/68

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Y	page 193, column 1, paragraph 2 -column 2, paragraph 2 page 195, column 1, paragraph 2 --- -/--	1-8, 12-54



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

14 August 2002

Date of mailing of the international search report

14. 11. 02

Name and mailing address of the ISA

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Authorized officer

Keller, Y

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 01/15093

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAI LAN PIAO ET AL: "An Arabidopsis GSK3/shaggy-like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and Absciscic Acid" PLANT PHYSIOLOGY, vol. 119, April 1999 (1999-04), pages 1527-1534, XP002181564	1-8
Y	abstract  page 1528, column 1, paragraph 3 -column 2, paragraph 3 figures 1,7,9 page 1531, column 1, paragraph 3 -page 1532, column 2, paragraph 1	1-8, 12-54
X	TATJANA KLEINOW ET AL: "Functional identification of an Arabidopsis snf4 ortholog by screening for heterologous multicopy suppressor of snf4 deficiency in yeast" THE PLANT JOURNAL, vol. 23, no. 1, 2000, pages 115-122, XP002181565 abstract page 115, column 1, paragraph 1 -column 2, paragraph 2 table 1 figure 2 page 119, column 2, paragraph 2 -page 1120, column 1, paragraph 1 page 120, column 2, paragraph 2 -page 121, column 1, paragraph 1	1-3
X	DATABASE EMBL 'Online! Acc. No Y11526, 2 November 1998 (1998-11-02) XP002181566 the whole document	4-20
T	I. WINICOV: "New molecular approaches to improving salt tolerance in crop plants" ANNALS OF BOTANY, vol. 82, 1998, pages 703-710, XP001007288	

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 01/15093

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-5, 12-53 partly and 6, 7, 54 compl.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5, 12-53 partially and 6, 7, 54 completely

use of a red beet casein kinase gene (SEQ ID No 1 and 6) and the corresponding constructs, DNAs proteins, in methods for conferring stress resistance to eucaryotic cells.

2. Claims: 1-5, 12-53 partially and 8, 9 completely

use of the red beet dydroorotase (SEQ ID No 2 and 7) and corresponding constructs, DNAs, proteins, in methods for conferring stress resistance to eucaryotic

3. Claims: 1-5, 12-53 partially and 10, 11 completely

use of a red beet translation initiation factor 1A gene (SEQ ID No 3 and 8) and corresponding constructs, DNAs, proteins in methods for conferring stress resistance to eucaryotic cells.

4. Claims: 1-5, 12-53 partially

use of a red beet gene (SEQ ID No 4 and 9) and corresponding constructs, DNAs, proteins in methods for conferring stress resistance to eucaryotic cells.

5. Claims: 1-5, 12-53 partially

use of a red beet gene (SEQ ID No 5 and 10) and corresponding constructs, DNAs, proteins in methods for conferring stress resistance to eucaryotic cells.